ROLE OF THE TAL1/SCL TRANSCRIPTION FACTOR IN DIFFERENTIATION OF BONE MARROW MONOCYTE-MACROPHAGE PRECURSORS

By

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Dedicated to the memory of my mother, Sanghamitra Dey

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LIST OF ABBREVIATIONS

5-FU: 5-fluorouracil

7-AAD: 7-aminoactinomycin D

AGM: Aorta-Gonad-Mesonephros

BFU-E: Blast Forming Unit-Erythroid

BM: Bone Marrow

CFC-Mk: Colony-Forming Cell-Megakaryocyte

CFU-C: Colony-Forming unit-Culture

CFU-GM: Colony-Forming Unit-granulocyte/monocyte

CFU-GEMM: Colony Forming Unit-granulocyte/erythrocyte/megakaryocyte/macrophage

CFU-M: Colony-Forming Unit-Monocyte

CLP: Common Lymphoid progenitors

CMP: Common Myeloid progenitors

Csf1r: Colony Stimulating Factor 1 Receptor

DMSO: Dimethyl Sulfoxide

EMSA: Electrophoretic Mobility Shift Assay

ES cells: Embryonic Stem Cells

HLH: Helix-Loop-Helix

HSC: Hematopoietic Stem Cell

IL-3: Interleukin-3

IL-6: Interleukin-6

LIF: Leukemia Inhibitory Factor

LKS: $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$

mAb: monoclonal antibody

MCP: Mast Cell progenitors

M-CSF: Macrophage Colony Stimulating Factor

MM: monocyte-macrophage

MPP: Multi-potential progenitors

NK cells: Natural Killer cells

PBS: Phosphate buffered saline

pc: *post coitum*

RT: room temperature

TPA: 12-O-tetradecanoylphorbol-13-acetate

T-ALL: T cell Acute Lymphoblastic Leukemia

CHAPTER I

INTRODUCTION

Hematopoiesis

Hematopoiesis is the process by which all types of blood cells are produced. Prenatally, hematopoiesis initiates in the extra-embryonic yolk sac where primitive (or embryonic) nucleated red blood cells are formed. At later stages of development, hematopoiesis shifts to the fetal liver, spleen and eventually to the bone marrow (BM). The other site of hematopoietic activity which functions in mouse ontogeny at the pre-liver stage of hematopoiesis is the aorta-gonad-mesonephros (AGM) region, where pluripotential hematopoietic stem cells (HSCs) are formed and get sublocalized to the dorsal aorta. Stem cells in the AGM region appear before the fetal liver, indicating the importance of this mesodermal region of the embryo in stem cell migration. After birth, hematopoiesis is restricted to the BM and daily blood cell production amounts to approximately 2.5 billion red cells, 2.5 billion platelets, and 1.0 billion granulocytes per kilogram of body weight. In the normal physiological system, the level of different types of blood cells are maintained within a range by a highly self-regulatory mechanism that is responsive to the demands put upon it. Under abnormal conditions like BM replacement or during excessive demand of blood cells due to anemia, extramedullary hematopoiesis is initiated in the liver or spleen.

Mature blood cells of different lineages are formed from the same primitive HSC in the bone marrow. The HSC in the BM has the capacity to proliferate, self-renew and

differentiate to lineage specific progenitor cells that further differentiate into mature cells that are released into the peripheral blood. Although a small percentage of this HSC population is cycling at any given time, daily production of 6 billion blood cells from this small population represents a massive amplification process.

Hierarchical organization of adult hematopoiesis

Hematopoiesis is a complex process in which a small number of HSCs expand and differentiate to form mature cells of different lineages that perform specific functions in the physiological system. Differentiation of progenitor cells into lineage specific cells results in loss of multi-lineage potential and follows a hierarchical pattern where HSCs in the BM give rise to both myeloid- and lymphoid-lineage specific cells through the formation of multi-potential progenitor (MPP) cells. This complex process of differentiation and lineage commitment is controlled by a collection of transcription factors, cytokines, cell adhesion molecules and external signals from the BM microenvironment. According to the classical model, the MPPs give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) and CLPs further differentiate into T cells, B cells and Natural Killer (NK) cells while CMPs differentiate into monocytes, granulocytes, megakaryocytes and erythrocytes (Fig. 1). Megakaryocytes and monocytes differentiate further into platelets and macrophages, respectively.

Over the past three decades, several models other than the classical model have been proposed. One of these is a stochastic model postulated by Ogawa and colleagues

(Ogawa et al., 1983). This model is based on the observation that various combinations of lineages can be found in the colonies of the colony forming unit in culture (CFU-C) assay, leading to the proposal that the combination of lineages occurs at random during the lineage restriction process. Therefore, this model is also called the 'random restriction' model. This proposal was originally restricted to myelo-erythroid lineages, but some researchers have interpreted this model to include lymphoid lineages.

In another model proposed by Brown and colleagues in 1985, multipotent progenitors were proposed to change their potential to make erythroid, myeloid, B and T cells, along with differentiation, and between each stage, bipotential progenitors would be produced (Brown et al., 1985; Brown et al., 2007). This so-called 'sequential determination' model incorporates the notion that B cells and macrophages are closely related, which does not explain the finding that T cell progenitros that have terminated B cell potential still retain myeloid potential.

In 1996, a different model was proposed by Singh and colleagues based on findings in mice deficient for certain transcription factors. In this model, the erythroid branch from the HSC occurs first, and the resulting CMLP subsequently generates a myeloid progenitor and CLP (Singh, 1996). In this regard, the order of developmental options is similar to the sequential determination model described above. This model was primarily based on the following two findings: (i) in PU.1$^{-/-}$ mice, erythroid cells are formed normally but myeloid, T and B cells are defective (Scott et al., 1994); and (ii) Ikaros$^{-/-}$ mice exclusively lack T and B cells (Georgopoulos et al., 1994). However, later studies reported that T cell development was not so strongly affected in these cases (Wang et al., 1996; Spain et al., 1999). Moreover, the lack of multiple lineage cells in a

knockout mouse does not necessarily prove the presence of common progenitors for them.

Irrespective of hematopoiesis differentiation model, a critical feature of the HSC is to balance between apoptosis, self-renewal and differentiation and must express genes that enable it to make that choice. Moreover, lineage specification requires up-regulation of genes associated with the adopted pathway and silencing of genes that specify the alternate pathways. This unique gene expression profile in the lineage-specific mature cells enable them to perform critical physiological functions and this whole process of hematopoietic cell differentiation and function is tightly regulated by the action of several different transcription factors and growth factors.

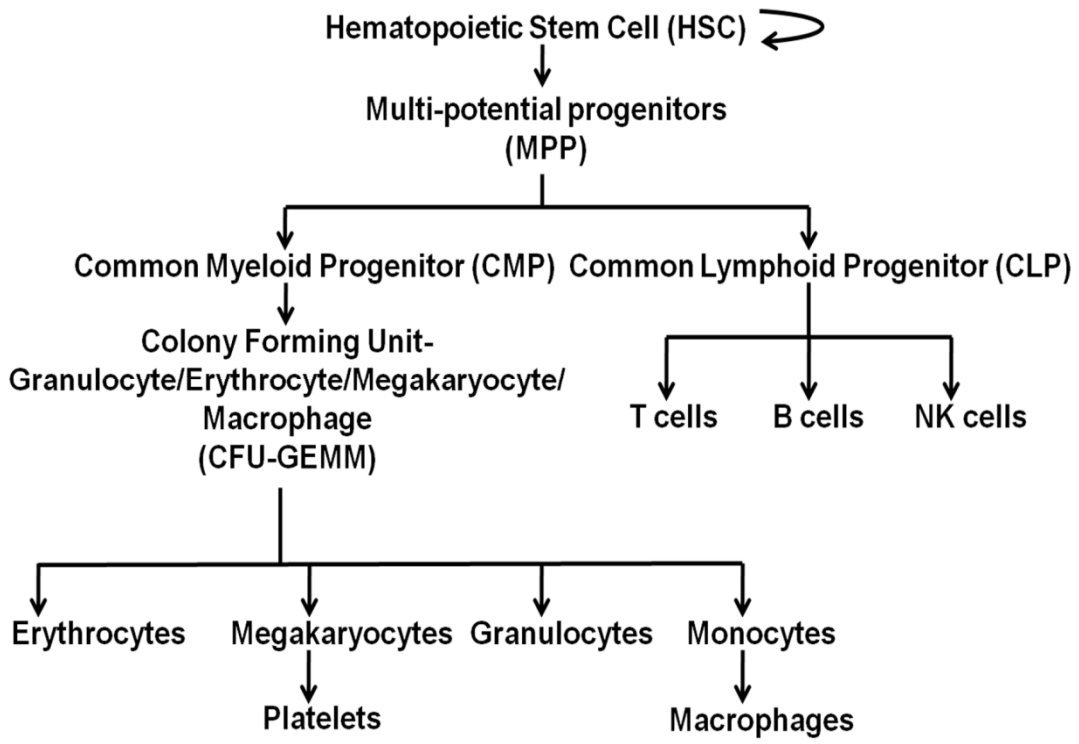

Figure 1. Formation of lineage committed mature cells from parent HSCs through formation of MPPs. HSCs serve as parent cells that can self-renew and also differentiate to form MPPs. The MPPs further diverge to form CMPs and CLPs; while CMPs form erythrocytes, megakaryocytes (platelets), granulocytes and monocytes, CLPs give rise to lymphoid lineage cells which are T-cells, B-cell and natural killer (NK) cells.

Monocytopoiesis

Monocytes or macrophages act as critical effectors and regulators of innate immunity and inflammatory responses. These cells represent a subgroup of leukocytes or white blood cells (WBCs) originally described as a population of BM-derived myeloid cells that circulate in the blood as monocytes and populate tissues as macrophages in the steady state and during inflammation (van Furth and Cohn, 1968). Monocytopoiesis, or formation of monocytes, is a part of the myeloid differentiation system with CMPs giving rise to colony forming units-granulocyte/monocyte (CFU-GM) that sequentially differentiate into colony forming units-monocyte (CFU-M), monoblasts, promonocytes and finally monocytes (Fig. 2). Monocytic differentiation is driven by cytokines including interleukin-3 (IL-3) and macrophage colony stimulating factor (M-CSF or colony stimulating factor 1, CSF-1), and is dependent on expression of the CSF-1 receptor (CSF1R, also known as C-FMS, M-CSFR and CD115), a member of the CSF1/PDGF receptor family of transmembrane tyrosine-protein kinases (Cecchini et al., 1994; Dai et al., 2002; Sasmono et al., 2003). Other important monocyte-macrophage (MM)-specific surface proteins include Fcγ receptor (II/III), macrosialin (CD68), scavenger receptor (SR), F4/80, CD14, CD11b and CD18. Monocytes, once formed in the BM, enter the blood stream and can migrate from blood into tissues with the help of chemokine receptors and adhesion receptors. While peripheral blood monocytes are able to perform phagocytosis and cytokine secretion, these functions are further enhanced with its differentiation into tissue macrophages, which participates in innate and adaptive immunity and helps in the maintenance of tissue homeostasis.

Transcription factors play critical roles in monocytopoiesis. Ectopic expression of several transcription factors like MafB, c-Maf, Egr1, ICSBP/IRF8, KLF4, E2-2 and PU.1 in early progenitors drives MM differentiation (Auffray et al., 2009). PU.1, an Ets-family member has key selective functions at several branch points of myeloid lineage diversification, particularly during lineage commitment towards either macrophage or dendritic cells (Bakri et al., 2005). Inhibitory interactions of PU.1 to repress GATA-1 and GATA-2 expression is required for blocking the commitment towards the erythroid/megakaryocytic and mast cell lineages respectively (Walsh et al., 2002). As a downstream target of PU.1, KLF4 can also induce macrophage fate and can selectively rescue monocyte differentiation of $PU.1^{-/-}$ progenitors, whereas KLF4 deficiency biases myeloid progenitor differentiation towards the granulocytic fate (Feinberg et al., 2007). At later differentiation stages, intermediate expression of PU.1 in GMPs can overcome the neutrophil fate-inducing effects of the basic leucine zipper transcription factor C/EBPα and activate the macrophage-specifying zinc finger transcription factors Egr-1 and Egr-2 (Laslo et al., 2006; Auffray et al., 2009). Similarly, introduction of the transcription factor ICSBP/IRF-8 in ICSBP/IRF-8-deficient myeloid progenitors resulted in monocytic differentiation at an expense of granulocytic differentiation (Tamura et al., 2000). These studies suggest that a balance between agonistic and antagonistic transcription factors that drive alternative fates is critical for monocyte differentiation.

In summary, monocytopoiesis is a critical and complex process that is highly regulated by different transcription factors that can in turn couple with cytokine receptor signaling pathways during lineage commitment. The functional aspect of monocyte-macrophage cells are further controlled by cell migratory stimuli from the inflammatory

milieu, expression of chemokine and adhesion receptors, and pathogen-associated pattern recognition receptors. Although studies done over the years have shed light on this process, transcription factors that control MM subtype-specific programs need to be identified. Models that closely reproduce defined differentiation stages in culture and development of new genetic tools of gene expression regulation could accelerate discovery in this area.

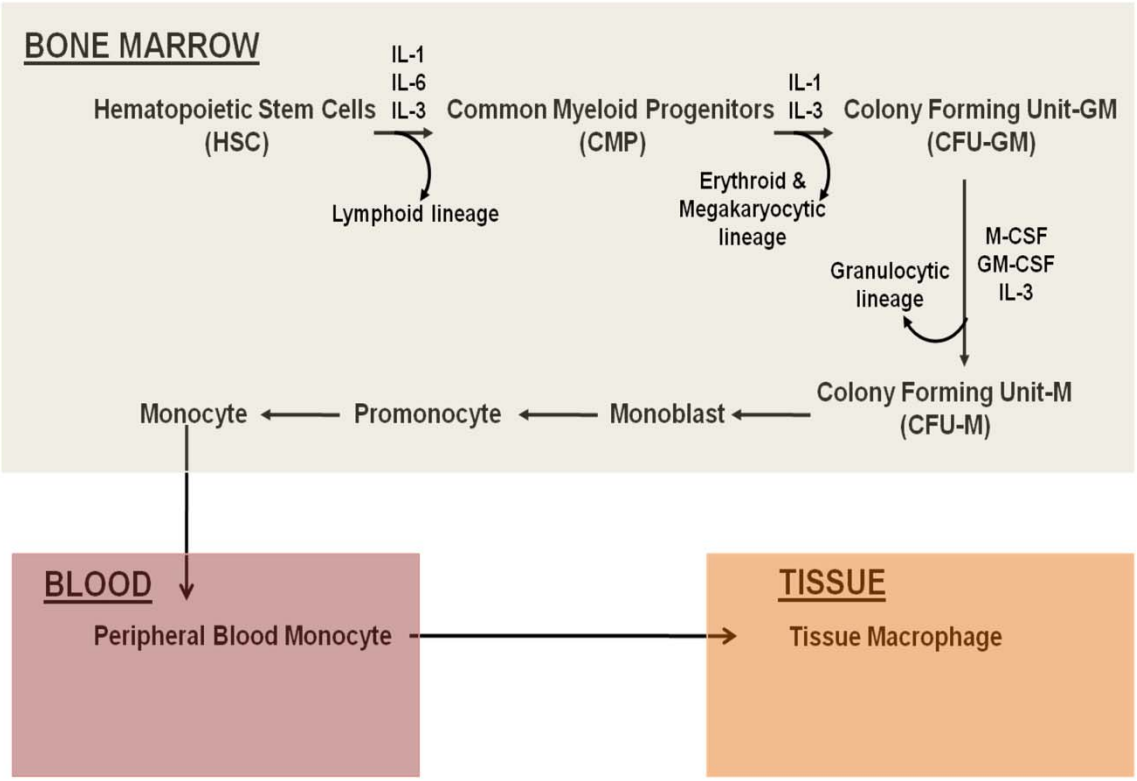

Figure 2. Schematic representation of differentiation of HSCs towards peripheral blood monocytes and finally tissue macrophages. Cytokines and growth factors required are shown adjacent to the arrows which point towards more differentiated cell types. Cells become lineage restricted with differentiation and alternative lineages diverge away from monocyte-macrophage lineage. Monocytes are released into blood stream and reach tissue sites where they develop into the most mature phagocytic cells.

T cell Acute Leukemia 1 (TAL1)

The *TAL1* gene, also known as *stem cell leukemia* (*Scl*), encodes a class II basic helix-loop-helix (bHLH) transcription factor, 47 kD in size, and was first identified as aberrantly expressed in 3% of human T cell acute lymphoblastic leukemia (T-ALL) cases. This results from a t(1;14)(p34;q11) translocation, that cleaves the *TAL1* gene on chromosome 1, separating its 5' end from the rest of the gene that is transposed into the T cell receptor α/δ locus on chromosome 14 (Carroll et al., 1990). In another 12-26% of T-ALL cases, interstitial deletion of approximately 90-100 kb region of chromosome 1 puts the promoter region of an upstream gene, *SCL interrupting locus* (*SIL*) in direct contact with the coding region of *TAL1* (Aplan et al., 1990; Brown et al., 1990; Bernard et al., 1991). Taken together, gain-of-function mutations in *TAL1* contribute to approximately 30% of T-ALL cases.

TAL1 recognizes E box sequences (CANNTG) in gene regulatory regions in DNA, and binds these sequences as a heterodimer with class I bHLH transcription factors termed E proteins (Hsu et al., 1991; Doyle et al., 1994; Hsu et al., 1994b). E proteins that interact with TAL1 are either products of the *E2A* gene, E12 and E47, or related protein E2-2 or *HEB/HTF4* gene products. Interaction of TAL1 with other factors is mediated by its HLH domain and sequence-specific DNA binding is mediated by its basic region. The preferred sequence that is recognized by this heterodimer is CAGATG with further preferences on either side of the E box governed by the associated interacting E protein (Hsu et al., 1994a). In erythroid cells, a larger multimeric protein complex consisting of TAL1, E2A, LMO2, GATA-1 and Ldb1 binds to a different E box (CAGGTG) combined with a GATA site separated by 9-12 bp (Wadman et al., 1997). In $CD4^{-}CD8^{-}$ thymocytes

from *Lmo2* transgenic mice, instead of an E box-GATA motif, two different E boxes (CATCTG and CAGRTG), spaced approximately 10 bp apart, were found to be occupied by two Tal1-E protein heterodimers bridged together by a pair of LMO1/2 and Ldb1 molecules (Grutz et al., 1998).

Transcriptional Regulation by TAL1

TAL1 regulates transcription of target genes by forming a heterodimer with E protein binding partner and results in maximum transcriptional activation in erythroid cells in association with zinc finger GATA transcription factor family and LMO2 and Ldb1 (Wadman et al., 1997). This correlated with formation of two high molecular weight protein-DNA complexes in gel-shift assay. CAT reporter assays done in T cells showed transactivation of a minimal *thymidine kinase* promoter linked to four copies of preferred E box sites by TAL1, either LMO1 or LMO2 and also GATA-3, despite the absence of a GATA-binding site (Ono et al., 1997). In T cell-specific transcriptional activation of the *retinaldehyde dehydrogenase 2* (*RALDH2*) gene, TAL1 and LMO1/2 can act as cofactors to GATA3 that binds a single GATA site in a cryptic promoter that is indispensable for *RALDH2* expression (Ono et al., 1998). In this situation, direct DNA-binding ability of TAL1 is not required to regulate transcription of a target gene. In murine erythroleukemia (MEL) cells, the multimeric protein complex consisting of TAL1, E47, LMO2, Ldb1, SSBP2 and GATA-1, transactivates *Protein 4.2* (*P4.2*) expression by directly binding two E box-GATA motifs in its promoter region (Xu et al., 2003; Xu et al., 2007). A similar complex comprised of TAL1, E47, LMO2, Ldb1 and

GATA-1, also activated the *Glycophorin A* promoter in a human bipotent cell line TF-1 (Lahlil et al., 2004).

In addition to its function as a transcriptional activator, TAL1 can also act as a transcriptional repressor. It repressed transcription from artificial promoters containing preferred E box sequences (CAGATG) (Hsu et al., 1994a), E boxes from the μE2 and κE2/μE5 immunoglobulin enhancers (Doyle et al., 1994; Voronova and Lee, 1994; Hofmann and Cole, 1996), and also a MyoD binding site (Goldfarb and Lewandowska, 1995; Hofmann and Cole, 1996). LMO1 also increased TAL1 mediated repression of immunoglobulin μE5, μE2 and μE3 E boxes (Chervinsky et al., 1999) and LMO1 and LMO2 potentiated TAL1-mediated repression of the CD4 proximal enhancer and enforced expression of TAL1 and LMO1 inhibited T cell differentiation (Herblot et al., 2000). Moreover, TAL1 interacted with the nuclear co-repressor mSin3A and histone deacetylase 1 (HDAC1), both *in vitro* and *in vivo*, to repress transcription of an E box-containing promoter and a GAL4 reporter linked to a thymidine kinase minimal promoter (Huang and Brandt, 2000). This repression was relieved by the action of a specific histone deacetylase inhibitor trichostatin A (TSA), suggesting that this TAL1-corepressor complex inhibits gene expression. The SWI/SNF protein Brg1 was associated with the TAL1-containing protein complex at *P4.2* promoter region in uninduced MEL cells and the association decreases with terminal erythroid differentiation and increased P4.2 expression (Xu et al., 2006). Lastly, the transcriptional co-repressor ETO-2 interacts with TAL1 along with another repressor, Gfi-1b, in early stages of erythroid differentiation and this association is lost during erythroid differentiation (Schuh et al., 2005; Cai et al., 2009).

Function of Tal1 in embryonic development

During mouse embryonic development, *Tal1* expression can be detected in embryonic and extraembryonic mesoderm at embryonic day 7.5 *post coitum* (*pc*), followed by endothelial and hematopoietic cells of yolk sac blood islands at day 8.5 *pc*, and neural tube and fetal liver at day 9-10.5 *pc* (Kallianpur et al., 1994). The *TAL1* gene is also expressed during early stages of human embryogenesis in aorta associated $CD34^+$ hematopoietic cells and at later stages in hematopoietic progenitors in fetal liver and bone marrow (Labastie et al., 1998). In zebrafish embryos, Tal1 is expressed in early hematopoietic and endothelial progenitor cells and also at definitive hematopoiesis initiation sites (Gering et al., 1998; Zhang and Rodaway, 2007). Gene knockout studies in mice have identified critical functions performed by TAL1 during embryonic development. *Tal1*$^{-/-}$ mouse embryos lack yolk sac erythropoiesis and failed to produce hematopoietic progenitor cells in general, which lead to embryonic lethality at day 9.5 *pc* (Robb et al., 1995; Shivdasani et al., 1995). Embryoid bodies derived from *Tal1*$^{-/-}$ embryonic stem (ES) cells fail to generate hematopoietic colonies of any type (Porcher et al., 1996) and did not express genes encoding key hematopoietic transcription factors like *GATA-1*, *PU.1*, *EKLF* and also lacked *globin* and *myeloperoxidase* mRNA expression (Robb et al., 1996; Elefanty et al., 1997). Chimeric mice generated by injecting *Tal1*$^{-/-}$ ES cells into both wild-type and *RAG2*$^{-/-}$ blastocysts showed no contribution by *Tal1*$^{-/-}$ ES cells to any hematopoietic lineage (Porcher et al., 1996). Apart from its role in hematopoiesis, TAL1 expression in embryonic endothelium is critical for angiogenic remodeling (Visvader et al., 1998) and is essential for driving GATA2 expression by binding an intronic enhancer element (Khandekar et al., 2007).

Function of Tal1 in postnatal development

In the adult hematopoietic system, TAL1 is expressed in HSCs and multipotent progenitors, including murine erythroid, megakaryocytic, myeloid and mast cell lineages (Visvader et al., 1991; Quesenberry et al., 1996; Begley and Green, 1999). Knockout of *Tal1* at the HSC stage adversely affected erythropoiesis and megakaryopoiesis leading to complete loss of blast forming unit-erythroid (BFU-E) and reduced colony forming cell-megakaryocytes (CFC-Mk) (Hall et al., 2003; Mikkola et al., 2003). In contrast, enforced expression of TAL1 in human $CD34^+$ cells resulted in increased numbers of erythroid and megakaryocytic progenitors (BFU-E and CFC-Mk) and an increase in the size of erythroid colonies (Elwood et al., 1998; Valtieri et al., 1998). A *LacZ* reporter gene 'knocked-in' to the TAL1 coding region revealed TAL1 promoter activity in progenitors of the erythroid lineage, B- and T-cell lineage, myeloid lineage and in multipotent progenitor cells capable of forming spleen colonies in irradiated mice ($CFU\text{-}S_{12}$) (Elefanty et al., 1998).

During *in vitro* differentiation of $CD34^+lin^-$ human cord blood cells towards the erythroid lineage, TAL1 is abundantly expressed in early erythroid progenitor cells (Ziegler et al., 1999) with levels peaking at the CFU-E stage (Condorelli et al., 1995) but decreasing with terminal erythroid differentiation (Cheng et al., 1996). In the murine erythroleukemia (MEL) cell line, Tal1 protein expression increases with terminal erythroid differentiation, and a Tal1-containing protein complex activated *Protein 4.2* (*P4.2*) gene expression by binding its proximal promoter region (Xu et al., 2003; Xu et al., 2007). A similar complex was also found to regulate *Glycophorin A* (*GPA*) gene expression by binding to the *GPA* promoter region in human bipotent TF-1 cell line

(Lahlil et al., 2004). Other potential TAL1 targets in this lineage include genes encoding for transcription factors GATA-1 (Vyas et al., 1999) and EKLF (Anderson et al., 2000). Although conditional gene knockout studies in adult mice have shown that *Tal1*-null mice develop anemia and Tal1 is required for normal erythropoiesis, it is not essential for adult erythropoiesis and does not significantly impair the ability of mice to respond to hemolytic stress (Hall et al., 2005).

Knockout studies done on mice have also identified a critical role of Tal1 in megakaryopoiesis and *Tal1*-null mice exhibit impaired platelet production along with megakaryocytic ultrastructural defects (McCormack et al., 2006). Gene expression analysis identified two target genes of Tal1 that encode for transcription factors NF-E2 and Mef2C, and in megakaryoblastic cell lines, bound the promoter regions of both the genes (McCormack et al., 2006; Gekas et al., 2009).

TAL1 is also expressed in mature mast cells and is required for proper differentiation of mast-cell progenitor cells (MCP) from the megakaryocyte-erythrocyte restricted cell fraction (Salmon et al., 2007). MCPs lacking *Tal1* gene showed increased expression of *Gata2* and reduced expression of IgE receptor and mast-cell proteases, MCP5 and MCP6 (Salmon et al., 2007).

TAL1 expression was also detected during adult vasculogenesis in mouse models of both physiological and pathological blood vessel and lymph vessel formation (Tang et al., 2006). Knockdown of *TAL1* in human primary endothelial cells repressed *in vitro* tubulomorphogenesis by reduction in *vascular endothelial* (*VE*)-*cadherin* gene

expression and an E box-GATA sequence in the *VE-cadherin* promoter region was found to be bound by TAL1, E47, LMO2 and GATA2 (Deleuze et al., 2007).

Function of Tal1 in monocytopoiesis

Although an important role for TAL1 has been demonstrated in other hematopoietic lineages, a function in mononuclear phagocyte production, while suggested by a number of observations, had not been determined. First, the mouse *Tal1* cDNA was cloned from a BM macrophage cDNA library using oligonucleotides derived from human HLH sequences (Begley et al., 1991). TAL1 interacts with specific E proteins to form productive DNA-binding complexes in the M1 monocytic leukemia cell line (Voronova and Lee, 1994). Although studies with this cell line and the human bipotent cell line TF-1 suggested that it was down-regulated during monocytic differentiation (Tanigawa et al., 1993; Voronova and Lee, 1994; Hoang et al., 1996), *TAL1* mRNA or protein has been detected in mouse peripheral blood mononuclear cells and BM macrophages (Kallianpur et al., 1994), and zebrafish (Zhang and Rodaway, 2007) and human (Pulford et al., 1995) macrophages. While deletion of *Tal1* in adult HSCs did not affect granulocyte-macrophage (GM) or macrophage progenitor cells numbers (Hall et al., 2003; Mikkola et al., 2003), double knockout of genes encoding bHLH transcription factors Lyl1 and Tal1 resulted in a ten-fold reduction of myeloid colonies formed from $lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ (LKS) cells cultured *in vitro* (Souroullas et al., 2009). Moreover, 5-fluorouracil (5-FU) treatment of $Tal1^{-/-}$ mice resulted in a small but significant delay in recovery of circulating monocytes (personal communication, Dr. David Curtis, The Royal Melbourne Hospital, Australia). Finally, knockdown of TAL1 in

human cord blood $CD34^+$ cells by short hairpin RNA (shRNA) reduced the numbers of both erythroid and myeloid cell progenitors (Brunet de la Grange et al., 2006).

Aims of Dissertation

The aims of this dissertation were:

1) **To evaluate *Tal1* gene expression during murine monocytopoiesis.**

The first aim was to critically study *Tal1* gene expression during differentiation of monocytes towards macrophages. TAL1 expression was detected in both mouse and human tissue macrophages, but a more detailed study was needed. To that end, TAL1 expression was determined at successive stages of monocytopoiesis from CMP cells to monoblasts, promonocytes, macrophages and finally in activated macrophages. CMP cells from mouse BM were isolated using a previously described procedure (Tagoh et al., 2002) and induced to differentiate towards macrophages by an *in vitro* culture system. Verification of this macrophage differentiation system was first carried out by morphological, immunocytometric and gene expression analysis of cells in the culture system.

2) **To study phenotypic consequences of *Tal1* gene manipulation in mouse BM monocyte-macrophage (MM) precursor cells.**

The second aim of the study was to define the biological role of Tal1 in this lineage by both over-expressing and knocking out *Tal1* gene expression in MM precursor cells. A previously established protocol of isolating monoblasts and promonocytes from mouse BM was utilized and the effects of *Tal1* gene manipulation was analyzed in an *ex*

vivo culture system. This approach not only enabled analysis of the role of Tal1 in a relatively pure population of BM cells but also facilitated efficient gene knockout using the Cre-Lox system. In the absence of a promoter that could drive *Cre* expression in a cell- and stage-specific manner that could have enabled *in vivo* gene knockout, *Cre* was delivered retrovirally in MM precursor cells harboring *loxP* sequences flanking *Tal1* locus and the same vector was used to over-express *Tal1* in wild-type (WT) cells.

3) **To identify Tal1 target genes and potential binding sites for Tal1 in regulatory regions of those genes.**

The final objective was to complement the phenotype analysis in *Tal1* knockout and over-expressing cells with gene expression analysis. The genes that are altered in expression could be direct or indirect targets of Tal1 and may explain the phenotypic changes seen with altered levels of Tal1 expression. Genes that are affected may have binding sites for Tal1 in their regulatory regions and we aimed at identifying those sites by molecular assays in both primary BM cells and a myeloid leukemia cell line.

CHAPTER II

MATERIALS AND METHODS

Cell lines and cell culture reagents

The connective tissue cell line L-929, mouse myeloid leukemia cell line M1, mouse fibroblast cell line NIH3T3, and retroviral packaging cell line BOSC23 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). L-929 cells were cultured in Eagle's Minimum Essential Medium (MEM) (Invitrogen), 15% FBS, 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Fisher Scientific, Pittsburgh, PA) and 1% penicillin-streptomycin. M1 cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin (Invitrogen) and their differentiation induced with 50 ng/ml of mouse interleukin-6 (mIL-6) (Stem Cell Technologies, Vancouver, Canada). The NIH3T3 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) (Invitrogen), 10% FBS, and 1% penicillin-streptomycin. The BOSC23 cell line was maintained in DMEM, 10% FBS, 1% penicillin-streptomycin, and GPT selection medium (Millipore, Billerica, MA).

L-cell conditioned media was used as a source of M-CSF during culture of mouse MM precursors. For preparation of L-cell conditioned medium, L-929 cells were seeded at 1.25×10^5 cells in 30 ml of medium. Media was harvested after 1-1.5 weeks and centrifuged at 1500 × g for 20 min and then forced through a 40 μm filter to remove cellular debris.

Retroviral expression vectors and cDNAs

Murine stem cell virus (MSCV)-based bicistronic vectors MSCV-IRES-GFP, MSCV-IRES-GFP-Tal1, and MSCV-IRES-GFP-Tal1^{T192P} have been previously described (Persons et al., 1997; Huang et al., 2000). A *Gata2* cDNA was provided by Dr. J. Douglas Engel (University of Michigan, Ann Arbor, MI) and the *Cre* coding sequence was obtained from Dr. Lishan Su (University of North Carolina, Chapel Hill, NC). The MSCV-IRES-YFP vector was provided by Dr. Derek Persons (St. Jude Children's Research Hospital, Memphis, TN) and *Tal1*, *Gata2* or *Tal1*^{T192P} cDNA was subcloned into this vector using EcoRI and XhoI restriction sites.

Mice, genotyping and Tal1 gene knockout in MM precursor cells

Wild-type 3 week-old C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). Mice with a *loxP*-targeted *Tal1* allele (*SCL*$^{loxP/loxP}$) and *SCL*$^{LacZ/WT}$ mice, both in a C57BL/6 background, have been previously described (Elefanty et al., 1998; Hall et al., 2003). The *SCL*$^{loxP/loxP}$ mice have loxP sequences flanking a 5' non-coding region of exon VI and the 3' UTR of the *Tal1* gene. The *SCL*$^{LacZ/WT}$ mice have a LacZ 'knock-in' cassette replacing exons IV, V and part of exon VI of *Tal1* gene locus, hence rendering that allele inactive while the other allele is wild-type. All mice were housed in an AAALAC accredited animal facility at Vanderbilt University Medical Center according to approved guidelines. *SCL*$^{loxP/loxP}$ and *SCL*$^{LacZ/WT}$ mice were intercrossed to generate *SCL*$^{loxP/LacZ}$ mice, which were genotyped by PCR analysis of DNA extracted from tail biopsies. The forward primer for the floxed and wild-type *Tal1* alleles was TCCCAAGCCCAAAGATTTCCCCAATG and for the excised allele was

GCAAGCTGGATGGATCAACATGGACCT. The reverse primer for all three alleles was GCAAGCTGGATGGATCAACATGCACCT. For detection of the *Tal1* LacZ knock-in allele, the forward and reverse primer sequences were respectively, GGATGGCGGGGCACACGAGGTAA and TGCCAGTTTGAGGGGACGACGACA. BM cells extracted from $SCL^{loxP/LacZ}$ mice were transduced with either MSCV-GFP-Cre or its parental vector using the methods described below. Cre expression resulted in excision of the single floxed *Tal1* locus, generating $SCL^{\Delta/LacZ}$ cells that were effectively *Tal1* nullizygous, while compound heterozygous cells ($SCL^{loxP/LacZ}$ genotype) transduced with the parental vector served as a control. GFP-expressing cells were isolated in a fluorescence-activated cell sorter and returned to culture for subsequent studies.

Fluorescence-activated cell sorting (FACS) and Flow Cytometry analysis

Retrovirally transduced cells were resuspended at a concentration of 5×10^6 cells/ml in phosphate-buffered saline (PBS) and filtered through a 70 μm filter to remove aggregates and debris in the suspension. These cells were sorted in a FacsAria instrument (Becton Dickinson, Franklin Lakes, NJ) using FacsDiva software on the basis of green fluorescence protein (GFP), yellow fluorescence protein (YFP), or combined GFP and YFP fluorescence by comparison with mock-transfected cells. After sorting, cells were collected in complete culture media as described below.

Standard flow cytometry analysis was carried out in BD FacsCanto II instrument (Becton Dickinson), also running FacsDiva software and data analysis was carried out using WinList software and PKH26 dye dilution data were modeled with ModFit LT software (Verity Software House, Topsham, ME).

Isolation and culture of common myeloid precursors (CMPs) from mouse BM

Purification and culture of CMPs were carried out according to previously published procedures (Tagoh et al., 2002) with certain modifications. BM cells were isolated from 3-5 week-old C57BL/6J mice (Jackson Labs) and depleted of lineage-committed cells using a Mouse Lineage Cell Depletion kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's recommendations. Using this kit, lineage-positive cells were indirectly magnetically labeled using a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent and anti-biotin monoclonal antibodies conjugated to magnetic microbeads as secondary labeling reagent. The magnetically labeled lineage positive cells were depleted by passing through a magnetic field in LS column (included in the kit). Unlabeled, lineage marker-negative cells, that pass through the column, were washed with buffer (PBS containing 1% FBS) and incubated with anti-mouse CD16/CD32 Fc blocker (BD Biosciences, San Jose, California) for 5 min on ice. The cells were then stained with Pacific-Blue-conjugated anti-mouse CD117 (c-kit) (Biolegend, San Diego, CA), phycoerythrin (PE)-conjugated anti-Ly6C (Miltenyi Biotec) and allophycocyanin (APC)-conjugated anti-CD31 (eBioscience, San Diego, CA) monoclonal antibodies (mAbs) or their isotype controls. The c-kit^{high}/CD-31^{high}/Ly6C^{neg} population was then isolated by FACS and placed in culture containing DMEM, 15% FBS, 0.5 μM β-mercaptopethanol (Sigma-Aldrich, St. Louis, MO), 20 ng/ml recombinant mM-CSF, 15 ng/ml interleukin-3 (IL-3), and 100 U/ml interleukin-1-alpha (IL-1α) (all from Stem Cell Technologies) to induce macrophage differentiation. Macrophages were activated by addition of 150 U/ml interferon-γ (IFN–γ) (BioLegend) and 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich).

Retrovirus preparation

BOSC23 retrovirus-packaging cells (Pear et al., 1993) were transfected with MSCV-based vectors, using Lipofectamine 2000 (Invitrogen) following manufacturer recommended conditions with certain modifications. Briefly, 24 hr before transfection, 2 × 10^6 BOSC23 cells were suspended in 4 ml of antibiotic-free media and plated in 60-mm culture dishes. For transfection of each plate, 20 μl of Lipofectamine 2000 was mixed with 0.5 ml of Opti-MEM (Invitrogen), incubated for 5 min at room-temperature (RT), and mixed with another 0.5 ml of Opti-MEM containing 8 μg of DNA. The DNA-Lipofectamine 2000 mixture was incubated for an additional 20 min at RT and added to the cells on the plate. Subsequently, after 3-4 hr incubation at 37˚C, old media was replaced with 3.5 ml of fresh antibiotic-containing media and returned to culture for 48 hr. High-titer, ecotropic virus-containing conditioned medium was collected 48 hr after transfection, filtered through 0.45 μm filter and stored at -80˚C for later use.

Retroviral transduction of MM precursor cells

BM cells were isolated from 3-5 week-old mice and placed into culture in DMEM containing 15% FBS, 0.5 μM β-mercaptopethanol, 20 ng/ml recombinant mM-CSF, and 15 ng/ml IL-3. After 24 hr, non-adherent cells were subjected to Pronase digestion (Roche, Branford, CT) followed by centrifugation over a step gradient of horse serum (Hyclone, Logan, UT) (Tushinski et al., 1982; Guilbert and Stanley, 1986; Stanley, 1990). The mononuclear cells isolated were mixed with conditioned media containing viral particles and 4 μg/ml polybrene (Sigma-Aldrich) and subjected to two rounds of

centrifuge-assisted infection (9000 × g for 1 hr each at 37˚C) at an interval of 3-4 hr. Cells were returned to culture overnight and transduced a final time 12 hr later. After another 24 hr in culture, non-adherent cells were collected and analyzed for integration of the retroviral vector on the basis of GFP or YFP expression, and expressing cells were isolated and returned to culture in the presence of 20 ng/ml mM-CSF and 40% L-cell conditioned media.

Gene expression analysis by quantitative real-time PCR

Total cellular RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA) and genomic DNA removed by the DNA-Free kit (Ambion, Austin, TX). RNA was reverse-transcribed using iScript (Bio-Rad, Hercules, CA) and specific transcripts analyzed by real-time PCR using iQ SYBR Green Supermix (Bio-Rad). Gene expression was normalized to *ribosomal protein S16 (RPS16)* RNA expression. The sequences of the primers used were: *Tal1* forward primer, ATAGCCTTAGCCAGCCGCTC, *Tal1* reverse primer, GCCGCACTACTTTGGTGTGA, *RPS16* forward primer, CACTGCAAACGGGGAAATGG, *RPS16* reverse primer, CACCAGCAAATCGCTCCTTG, *Gata2* forward primer, CCGACGAGGTGGATGTCTTC, *Gata2* reverse primer, TGGGCTGTGCAACAAGTGTG, *p16(Ink4a)* forward primer, GGACATCAAGACATCGTGCGA, *p16(Ink4a)* reverse primer, ATTGGCCGCGAAGTTCCA, *m-Lys* forward primer, CTCTGGGACTCCTCCTGCTTT, *m-Lys* reverse primer, GCTAAACACACCCAGTCGGC, *Csf1r* forward primer,

CTAGCAAGCTGGTGCGGATT, *Csf1r* reverse primer,
AGCGTTGAGACTGAGAGCCC, *Pu.1* forward primer,
CGGATGTGCTTCCCTTATCAAAC, *Pu.1* reverse primer,
GGCGAATCTTTTTCTTGCTGC, *iNOS* forward primer
CCAAGATGGCCTGGAGGAAT, *iNOS* reverse primer
CCTGATGTTGCCATTGTTGGT.

Microarray analysis

BM MM precursor cells from wild-type mice were retrovirally transduced with either the MSCV-GFP-Tal1 vector or empty vector following the procedure described above, and GFP-expressing cells were sorted using FACS and put into culture as described above. Cells were cultured for 8 days and total cellular RNA was extracted using RNeasy kit (Qiagen) and genomic DNA removed by the DNA-Free kit (Ambion). Samples were submitted to the Vanderbilt Functional Genomics Shared Resource where quality control assays were carried out and cDNA was synthesized using 96-well Affymetrix one-cycle reactions (Affymetrix, Santa Clara, CA). The resultant cDNAs were then purified, and used as a template for subsequent *in vitro* transcription (IVT) reaction for complementary RNA (cRNA) amplification, labeled and hybridized onto GeneChip® Mouse 430 2.0 arrays (Affymetrix). Signal from empty vector transduced cells were used as baseline and genes that changed more than or equal to two-fold in *Tal1* transduced cells were analyzed for pathway analysis using Ingenuity Pathway Analysis software (Redwood City, CA).

Apoptosis analysis

MM precursors from different times in culture were stained with APC-conjugated Annexin V (BD Biosciences) and the DNA-intercalating dye 7-aminoactinomycin D (7-AAD) (Invitrogen) and analyzed by flow cytometry. Cells that excluded all stains were scored as viable. Briefly, the cells were suspended in 100 μl of binding buffer (10 mM Hepes buffer, pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride), and pulsed for 15 min at room temperature in the dark with 5 μl of APC-Annexin V. After washing, each sample was resuspended in 500 μl of binding buffer, stained with 2 μg of 7-AAD, and analyzed by flow cytometry.

Cell division analysis by dye-dilution method

Cell division was analyzed with the membrane-intercalating red fluorescent dye PKH26 using the PKH26 MINI kit (Sigma-Aldrich). Retrovirally transduced cells were labeled with PKH26 according to the manufacturer's instructions, and cells positive for both PKH26 and GFP fluorescence were sorted and returned to culture. Cells stained with a single color (GFP or PKH26) were used for comparison. Cells in culture were analyzed at intervals by flow cytometry and the decrease in intensity of fluorescence over time in culture was used to quantify cell division. This was modeled using ModFit LT software.

Differentiation analysis of MM precursor cells

Differentiation of MM precursors was studied by flow cytometry analysis of cell surface antigens with PE-conjugated anti-Ly-6C (Miltenyi Biotec), APC-conjugated anti-

CD31 (eBioscience) mAbs and their isotype controls (Tagoh et al., 2002). Briefly, cells in culture were collected by centrifugation, washed with buffer (PBS with 1% FBS) and blocked for 5 min on ice with anti-mouse CD16/CD32 Fc blocker (BD Biosciences). Cells were then stained for 30 min with PE-Ly6C and APC-CD31 antibodies or isotype controls, washed, stained with 2 μg of 7-AAD and then analyzed by flow cytometry. Cell morphology was determined from Wright-Giemsa staining of cytospin preparations.

Cell cycle analysis

Bromodeoxyuridine (BrdU) pulse chase analysis was carried out using an APC BrdU Flow Kit (BD Biosciences) according to the manufacturer's recommendations. Cells were labeled with 10 μM BrdU for 45 min, washed with complete media and returned to culture containing fresh medium. Cells were collected at specific time points (0, 1, 2, and 4 hr), washed with staining buffer (1× Dulbecco's PBS, 3% FBS and 0.09% sodium azide), fixed in BD Cytofix/Cytoperm buffer (included in kit), and processed for flow cytometry analysis. Cells were also incubated with 10 μM BrdU for prolonged periods (12, 24, 36, and 48 hr). After labeling, these cells were processed for flow cytometry analysis exactly as above.

Flow cytometry analysis of DNA staining by Hoechst 33342 (Invitrogen) and RNA staining by Pyronin Y (Sigma-Aldrich) was used to differentiate cells in G0 from those in G1. In brief, 10 μg/ml of Hoechst 33342 was added to the cells and incubated for 45 min at 37˚C, after which cells were collected by centrifugation and fixed overnight at 4˚C in 5% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA). Then

next day, cells were stained with 2 μg/ml of Pyronin Y for 30 min on ice and finally resuspended in 5% PFA for flow cytometry analysis.

Chromatin Immunoprecipitation (ChIP) analysis

ChIP analysis was carried out using a commercial kit (Millipore) with certain modifications as described (Xu et al., 2003). Formaldehyde was added to 2×10^7 primary mouse BM cells at a final concentration of 1% for 20 min at 37˚C. For M1 cells, cross-linking was carried out with 1×10^8 cells that had been cultured in the presence or absence of recombinant mIL-6 for 48 hr. All subsequent steps were carried out according to the manufacturer's instructions. The antibodies used for immunoprecipitation of chromatin fragments included rabbit polyclonal anti-Tal1 (Kallianpur et al., 1994), anti-E47 (sc-763X, Santa Cruz), and anti-E2A (Yae) (sc-416X, Santa Cruz), as well as normal rabbit IgG (sc-2027, Santa Cruz). Immunoprecipitated DNA was analyzed by real-time PCR using iQ SYBR Green Supermix (Bio-Rad) and occupancy was quantified for all antibodies, including IgG, and expressed as percentage of input. The sequences of primers used were as follows: *p16(Ink4a)* -13 region forward, CCACTGGTCACACGACTG; *p16(Ink4a)* -13 region reverse, GCACGTCATGCACACG; *p16(Ink4a)* -2592 region forward, CCCTAGCCAAATCTGACAT; *p16(Ink4a)* -2592 region reverse, TCCAGGTAGTACATGCTTTCA; *p16(Ink4a)* -4638 region forward, CCTCTCCAGGGTAGTCATGG; *p16(Ink4a)* -4638 region reverse, GATGCAAGAATCACACAAAGG; *p16(Ink4a)* -5106 region forward, CACTTTGCTTATACTGGATGG; *p16(Ink4a)* -5106 region reverse,

CCAGTGAAACCCTTGTATCTT; *p16(Ink4a)* -6932 region forward, GCTTTCACAGGACACAGAAGG; *p16(Ink4a)* -6932 region reverse, TTACTCTTTCCCACCATGACC; *p16(Ink4a)* 3´ UTR forward, GGAGGGAGCAGAAGGAGG; *p16(Ink4a)* 3´ UTR reverse, ACAATCCAGCCATTATTCCC; *Gata2* intron 4 forward, CCTGCCGGAGTTTCCTATCC; *Gata2* intron 4 reverse, ACTGAGTCGAGGTGGCTCTGA; *Gata2* 3´ UTR forward, TCGATTCTGTGTGTGGTGGT; and *Gata2* 3´ UTR reverse ACTTCCGGTTAGGGTGCTCT.

Western Blot analysis

Western blot analysis was used to test Cre expression by MSCV-GFP-Cre vector and to test Tal1 expression in MM precursor cells isolated from wild-type BM. NIH3T3 cells were transfected with the MSCV-GFP-Cre vector or MSCV-GFP parental vector using Lipofectamine 2000 following manufacturer's recommendations, cultured for 48 hr, and followed by immunoblot analysis as described later. Whole cell lysates were also prepared from MM precursors at days 1, 4, 6 and 8 of culture. For preparation of whole cell lysate, cells were washed three times with ice-cold PBS and solubilized for 30 min in ice-cold RIPA buffer (Millipore) containing protease inhibitor cocktail (Roche). Cells were lysed by passing through a 23G needle, rotated end-to-end for 30 min at 4˚C and then centrifuged for 10 min to pellet insoluble material. The protein concentration of cleared lysates was quantified using the BCA reagent (Pierce).

Lysate proteins were separated electrophoretically using 10% Bis-Tris NuPAGE gels (Invitrogen) and transferred to 0.45 μm pore nitrocellulose membranes (BioRad). Membranes were blocked for 1 hr in 5% non-fat dry milk at RT and then incubated overnight with primary antibodies to either Tal1 (sc-12982, Santa Cruz) or β-actin (ab8226, Abcam, Cambridge, MA) or Cre (ab24607, Abcam) at 4˚C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at RT and proteins were visualized by enhanced chemiluminescence using the SuperSignal West Pico system (Pierce).

CHAPTER III

TAL1 EXPRESSION IN BM PRECURSORS DIFFERENTIATING TOWARDS MACROPHAGES

Introduction

Expression and specific functions of Tal1 have been extensively studied in hematopoietic lineages like erythrocytes, megakaryocytes, mast cell precursors and also in endothelial cells during vasculogenesis and lymphangiogenesis (Visvader et al., 1991; Quesenberry et al., 1996; Begley and Green, 1999). However, a detailed study of Tal1 expression during monocytopoiesis was needed, in part because studies in the TF-1 and M1 cell lines suggested Tal1 expression was down-regulated with differentiation. However, both these leukemic cell lines constitute artificial models in which differentiation is induced by IL-6 in the case of M1 cells and by 12-O-tetradecanoylphorbol-13-acetate (TPA) in TF-1 cells, whereas, physiologically, M-CSF and IL-3 are the cytokines responsible for monocyte differentiation. In the present study, *Tal1* mRNA expression was studied in primary CMPs differentiating *ex vivo* into macrophages and also in LPS/IFN-γ activated macrophages. Previously reported findings in this system, in terms of morphology, cell surface marker expression and expression of known macrophage-specific genes, were verified in cells generated with our hands. Finally, using a different strategy developed by Stanley and colleagues, Tal1 protein expression was detected in monoblasts and promonocytes isolated from mouse BM and also at subsequent differentiation stages.

Results

A. Tal1 mRNA expression during differentiation of CMPs to macrophages

CMPs were isolated by a previously described method (Tagoh et al., 2002) of enriching lin^- cells from mouse BM and purifying the c-kit^{high}/CD-31^{high}/$Ly6C^{neg}$ population. These cells were induced to differentiate to macrophages in presence of MM-specific growth factors. Using quantitative RT-PCR analysis, Tal1 mRNA expression was detected at all stages from CMPs to post-mitotic macrophages (Fig. 3A). Although *Tal1* message fluctuated in abundance, it was detectable throughout, as well as in LPS- and IFN-γ-activated macrophages, although at lower levels than unactivated cells (Fig. 3A).

B. Verification of macrophage formation by the ex vivo culture system used

Formation of macrophages using the *ex vivo* culture system used was verified by morphological analysis of Wright-Giemsa stained cytospin preparations (Fig. 3B). Cells at day 0 and 1 in culture were smaller and spherical, with the nucleus occupying the majority of the cell space, while with differentiation, the cells became larger, vacuolated and more heterogenous in shape. RT-PCR analysis of genes encoding for macrophage specific markers like *lysozyme* and *Csf1r* showed the expected increase with differentiation, while *inducible nitric oxide synthase* (*iNOS*) mRNA expression could be detected only in activated macrophages (Fig. 3C). Genes for transcription factors like *Gata2* and *Pu.1* were down-regulated (Tagoh et al., 2002) while *p16(Ink4a)* mRNA expression increased with differentiation (Fig. 3C). Bone-resorptive osteoclast cells are

formed from fusion of MM precursor cells and require the presence of receptor activator of nuclear factor κβ (RANK)-ligand and M-CSF. Osteoclasts are characterized by expression of *calcitonin receptor* (*CALCR*) gene expression, which could not be detected in these cells, excluding the formation of osteoclasts by the culture system used (data not shown). In addition, expression of cell surface antigens, CD31 and Ly6C, were also studied during the culture system. As described (Tagoh et al., 2002), expression of those two antigens changed sequentially from $CD31^{+}Ly6C^{-}$ CMPs to $CD31^{+}Ly6C^{+}$, $CD31^{-}Ly6C^{+}$, and $CD31^{-}Ly6C^{-}$ cells (Fig. 3D).

C. Tal1 protein expression in MM precursor cells

Finally, a more mature population of MM precursors comprised of monoblasts and promonocytes were enriched using a method developed by Stanley and colleagues (Tushinski et al., 1982; Guilbert and Stanley, 1986; Stanley, 1990). These cells were induced to differentiate *in vitro* using recombinant murine M-CSF. Western blot analysis with cellular extracts prepared at days 1, 4, 6 and 8 in culture showed modest changes in Tal1 expression during this period (Fig. 3E).

Conclusion

The above studies, for the first time, detected Tal1 expression in primary cells differentiating towards macrophages and also in activated macrophages. Quantitative gene expression analysis in CMPs to macrophages was complemented with more definitive Tal1 protein detection in a mature population of MM precursors. The level of Tal1 did not significantly change during the process, consistent with a biological function

for this transcription factor in this lineage. The explant model of monocytopoiesis was also verified by testing gene expression analysis and expression of cell surface antigens.

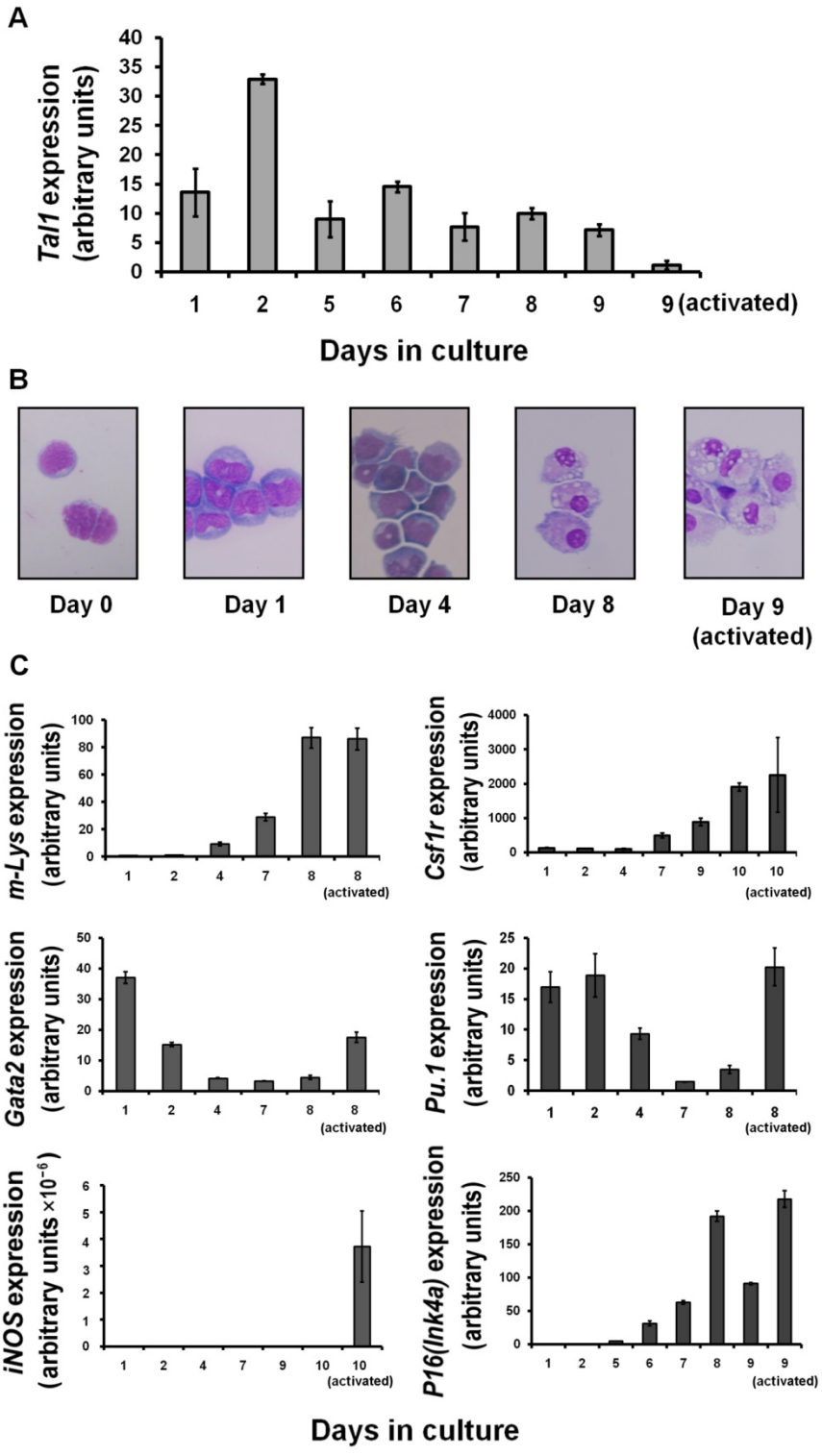

Figure 3—cont.

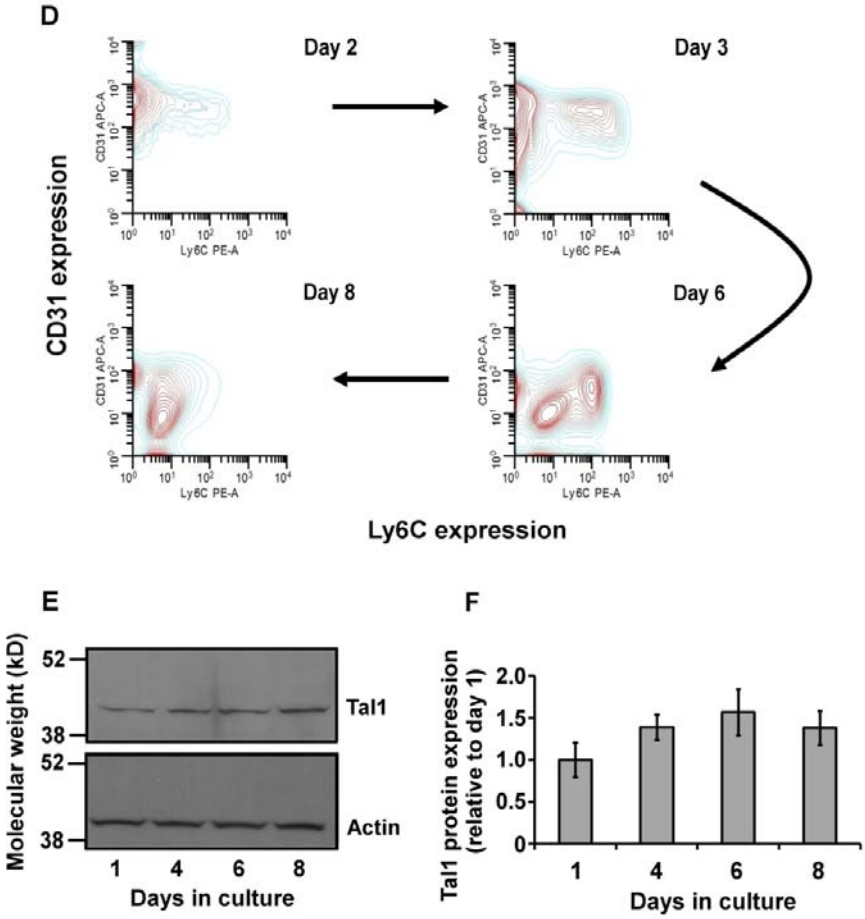

Figure 3. *Tal1* gene expression during *in vitro* differentiation of murine MM precursors to macrophages. **(A)** Total RNA prepared from purified precursors and differentiated cells at indicated times in culture were reverse transcribed for real-time PCR analysis employing SYBR Green fluorescence. Each bar represents mean ± SD from 3 independent PCR reactions as normalized to *RPS16* mRNA expression and is representative of three independent experiments. **(B)** Morphology of differentiating cells from Wright-Giemsa stains of cytospin preparations. **(C)** Gene expression analysis during *in vitro* differentiation of CMPs using quantitative real-time PCR. The genes tested were *lysozyme* (*m-Lys*), *colony stimulating factor-1 receptor* (*Csf1r*), *Gata2*, *Pu.1*, *inducible nitric oxide synthase* (*iNOS*) and *p16*(*Ink4a*), as expressed in arbitrary units relative to *RPS16* mRNA expression. Each bar represents mean ± S.D. of triplicate PCR experiments and is representative of three independent experiments. **(D)** Flow cytometry analysis of cell surface expression of CD31 and Ly6C during *in vitro* differentiation of CMPs. Arrows detail order of progression of differentiation. **(E)** Western blot analysis of Tal1 protein expression in terminally differentiating mouse BM MM precursors. Results are representative of two independent analyses. **(F)** Graph of normalized Tal1 expression from Western blot analysis.

CHAPTER IV

OVER-EXPRESSION OF TAL1 AND EFFECTS ON MONOCYTOPOIESIS

Introduction

Gain-of-function studies have been informative in defining transcription factor potential in driving particular cell-fate choices. Tal1 was continuously expressed during monocytopoiesis with the level of expression changing only modestly during this process. This suggested that there could be a specific role of this transcription factor in monocyte production, and we aimed to stud that by over-expressing Tal1. Ectopic expression of TAL1 in HSCs (Elwood et al., 1998) and hematopoietic progenitor cells (Valtieri et al., 1998) resulted in increased size and number of erythroid and megakaryocytic colonies and also stimulated proliferation in erythropoiesis (Valtieri et al., 1998). Moreover, heterodimerizing- and DNA binding-defective *TAL1*-mutants did not exhibit these effects (Valtieri et al., 1998), which suggested that direct protein or DNA interaction was required. Interestingly, although enforced expression of TAL1 resulted in inhibition of granulocytic and granulo-monocytic CFUs, it did not have any effect on the number of monocytic CFUs and markedly increased the number of primary and secondary macroscopic colonies containing monocytic cells (Valtieri et al., 1998).

In this study, *Tal1* cDNA was retrovirally delivered to a committed population of wild-type BM cells comprised of monoblasts and promonocytes, and gene expression was compared with cells transduced with an empty vector or a DNA binding-defective mutant of *Tal1*.

Results

A. Semi-quantitative and quantitative gene expression analysis

The MSCV-based retroviral vector MSCV-IRES-GFP was used to transduce BM MM precursor cells with cDNAs encoding wild-type Tal1 or a DNA binding-defective Tal1 mutant ($Tal1^{T192P}$). Empty vector-transduced cells were used as a control. Cells that expressed GFP were sorted using a cell sorter and put into culture and induced to differentiate in the presence of M-CSF. Gene expression analysis by semi-quantitative PCR for *Csf1r* and *Il6-r* transcripts showed up-regulation of both genes in Tal1 over-expressing cells (Fig. 4A). Interestingly, cells transduced with $Tal1^{T192P}$ cDNA did not show any difference in *Csf1r* or *Il6-r* mRNA abundance compared to vector-transduced control and suggested that up-regulation of at least these two genes in Tal1 over-expressing cells required direct DNA-binding. Expression of *CALCR* gene expression could not be detected in any of the three groups of cells (data not shown).

The monoblastic leukemia cell line, M1 can be induced to differentiate by IL-6, and previous studies revealed that with differentiation, Tal1 expression was abrogated along with loss of Tal1-E protein DNA-binding activity (Voronova and Lee, 1994). However, Tal1 over-expressing M1 cells, when induced to differentiate, showed an earlier and higher expression of *Csf1r* compared to cells transduced with either $Tal1^{T192P}$ cDNA or empty vector (Fig. 4B). Expression of *Il6-r* gene was also slightly higher in Tal1 over-expressing cells compared to control cells or cells expressing $Tal1^{T192P}$ (Fig. 4C). Moreover, expression levels of both genes were equivalent in empty vector-

transduced and *Tal1*^{T192P} transduced cells, suggesting that the DNA-binding ability of Tal1 was required for augmentation of *Csf1r* and *Il6-r* gene expression. Therefore, these findings were similar to those obtained from analysis of BM MM precursor cells.

B. Microarray analysis of genes up-regulated in Tal1 over-expressing cells

To assess differences in gene expression and function of MM precursor cells after Tal1 over-expression, a global gene expression profiling analysis was done using cDNA microarrays. Cells transduced with either empty vector or the MSCV-GFP-Tal1 virus were sorted and put into differentiation-inducing culture conditions for 8 days and finally used for isolation of total RNA. Arrays were hybridized with cDNAs prepared from these samples and analyzed for genes that showed greater than two-fold up-regulation in Tal1 over-expressing cells. Approximately 200 genes were expressed at a higher level compared to the control. Analysis of those 200 genes using Ingenuity software identified up-regulation of 68 different biological functions. Post-screening, relevant pathways that were significantly enhanced included immune response and antigen presentation functions and also pathways involving cell signaling, proliferation, cell cycling and gene expression (Fig. 5). Individual genes that participate in each pathway are shown in Table 1. This study suggests that Tal1 over-expression potentiates the differentiation machinery by enhancing proliferative potential and cell signaling in MM precursors. Moreover, increased antigen presentation and immunological activity suggest that Tal1 may also lead to functional enhancement of MM precursors.

Conclusion

In summary, these studies show that TAL1 has a positive role to play in MM differentiation. Over-expression of Tal1 in primary MM precursor cells potentiates the expression of macrophage-specific genes, which suggests that Tal1 co-operates with the differentiation process. Even for M1 cells in which Tal1 expression declines with progression of differentiation, Tal1 exerts a positive effect in the mechanism and does not block macrophage differentiation. Moreover, these effects of Tal1 were not seen in cells transduced with the DNA binding-defective mutant of *Tal1* (*Tal1*^{T192P}) suggesting that Tal1 binds DNA sequences to enhance gene expression. Expression of *CALCR*, which is a marker for osteoclast-specific differentiation, was also not detectable in either *Tal1*-transduced, *Tal1*^{T192P}-transduced or empty vector-transduced cells, suggesting that there was no shift in lineage commitment in these cells due to *Tal1* over-expression.

Finally, microarray gene expression studies have identified a potential enhancement of differentiation- and macrophage function-specific pathways and processes with Tal1 over-expression in this lineage. Previous studies have also identified similar up-regulation of immune response pathways on enhancement of Tal1 expression levels (Landry et al., 2008). However, *Tal1* mRNA levels were not elevated in activated macrophages (Fig. 3A), which suggests that this could also be secondary effects of increased cell signaling and cell surface receptor expression. Nevertheless, Tal1 over-expression studies do suggest an active role of Tal1 in MM precursors and point towards a critical function of this transcription factor in development of this lineage.

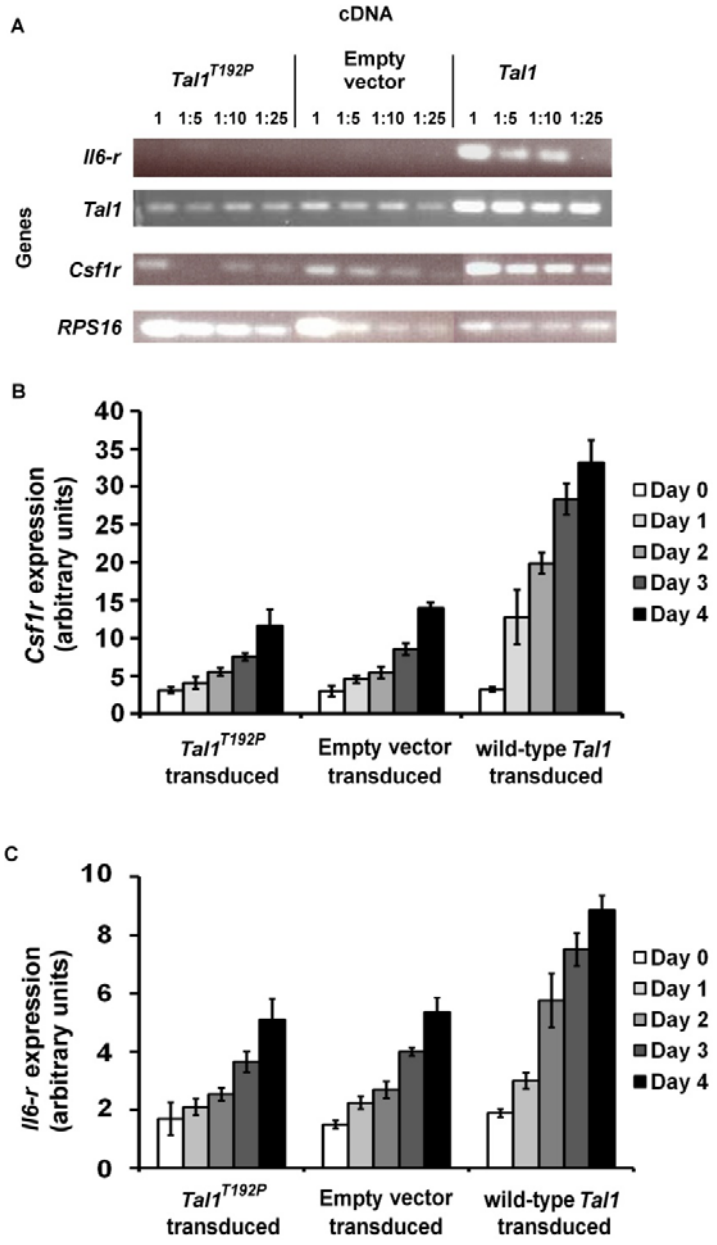

Figure 4. Comparative gene expression analysis in MM precursor cells **(A)**, and M1 cells **(B & C)** transduced with wild-type *Tal1* cDNA, *Tal1*^{T192P} cDNA and empty vector. **(A)** Total RNA extracted from day 3 MM precursor cells were reverse transcribed and serial dilutions of cDNA were analyzed by semi-quantitative PCR for 25 cycles with primers specific for *Tal1*, *Il6-r*, *Csf1r* and *RPS16* genes. PCR reaction products were resolved in a 2% agarose gel and a representative gel from three independent experiments is shown. M1 cells were cultured in presence of 50 ng/ml of IL-6 for 4 days and quantitative gene expression analysis was done for *Csf1r* **(B)** and *Il6-r* **(C)** genes at indicated time points. Each bar represents mean ± SD from 3 independent PCR reactions as normalized to *RPS16* mRNA expression. Results are representative of three independent experiments.

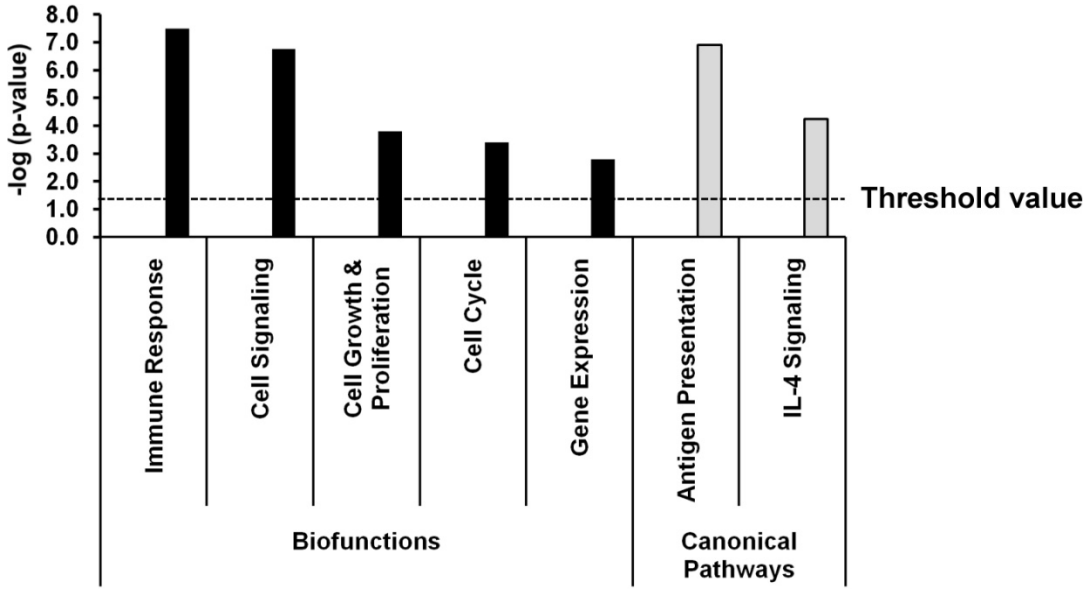

Figure 5. Results of pathway-analysis of microarray gene expression data. Critical pathways up-regulated in *Tal1*-transduced cells as identified by analysis of 200 genes that were found to be greater than two-fold increased compared to control. Dotted line shows threshold value for unaffected pathways.

Table 1. List of genes connected to pathways that were up-regulated in Tal1 over-expressing cells. Overlapping gene names denote role in multiple molecular functions.

Biofunctions or Pathways up-regulated	Genes in Network	-log(p-value)
(i) Immune Response	PARP1, HMGA2, GADD45A, TXNIP, CCND2, HLA-DOA, CD5, CCL22, HLA-DMB, ASS1, HLA-DOB, RUNX1, CSF1, IL1R2, PTGS2, CD86, BCL2L11, EBI2, LY6C1, HLA-DQB2, NR3C1, HLA-DRB1, CST7, CTSG, HLA-DQA1	7.5
(i) Cell Signaling	DNM2, PIP5K2B, MSN, PMAIP1, GDI2, CLEC5A, PARP1, RNF14, GADD45A, CCND2, PXN, HLA-DOA, RBPJ, CCL22, HLA-DMB, SDHA, HLA-DOB, EDNRB, PFN2, MMP10, RUNX1, PRKG1, IL1R2, SORBS1, PTGS2, ADAM10, H3F3B, BCL2L11, EBI2, TLK1, PPP1R12A, HLA-DQB2, NR3C1, HLA-DRB1, PPP3R1, F5, RCAN1, HLA-DQA1	6.75
(i) Cell Growth and Proliferation	PIP5K2B, DNM2, PMAIP1, ANGPTL6, PARP1, HMGA2, GADD45A, TXNIP, CCND2, RBPJ, CD5, ID2, DNAJA2, TRIM35, EDNRB, FSCN1, RUNX1, CSF1, PRKG1, CD86, PTGS2, ADAM10, BCL2L11, ERO1L, LY6C1, SEL1L, HLA-DQB2, NR3C1, HLA-DRB1, SERINC3, CTSG, PKP3, RCAN1	3.8
(i) Cell Cycle	PARP1, PTGS2, GADD45A, TXNIP, CCND2, BCL2L11, ID2, LY6C1, SEL1L, NR3C1, BRCC3, RUNX1, CSF1	3.4
(i) Gene Expression	CD86, PARP1, PTGS2, HMGA2, GADD45A, TXNIP, NFYA, CCND2, PXN, NPAS2, RBPJ, EBI2, ID2, SEC61A1, NR3C1, RUNX1, CSF1, RCAN1, PRKG1	2.8
(i) Antigen Presentation	HLA-DOB, HLA-DQB2, HLA-DRB1, HLA-DOA, HLA-DQA1, HLA-DMB	6.9
(i) IL-4 Signaling	HLA-DOB, NR3C1, HLA-DRB1, HLA-DOA, HLA-DQA1	4.25

CHAPTER V

TAL1 KNOCKOUT STUDIES IN MM PRECURSOR CELLS

Introduction

Tal1 expression and over-expression studies indicated an active role of this transcription factor during monocytopoiesis, both in terms of regulation of gene expression and maturation of MM precursors into macrophages. In addition to its expression during differentiation, Tal1 over-expression studies also revealed potentiation of macrophage-specific gene expression. On the other hand, a DNA binding-defective mutant of *Tal1* did not elicit any effect compared to control, and hence, it appears that Tal1 binding to target sequences in gene regulatory regions is necessary to regulate expression of its target genes. This suggests that a more detailed study of role of Tal1 in this lineage is required. A functional analysis of Tal1 can be done most effectively by using a strategy of knocking out *Tal1* and studying phenotypic effects in this lineage.

Inactivation of *Tal1* has been used effectively to understand its role in other hematopoietic lineages like erythrocytes, megakaryocytes and mast cell precursors (Hall et al., 2003; Mikkola et al., 2003; McCormack et al., 2006; Salmon et al., 2007). These studies have taken advantage of the Cre-Lox system of gene deletion where interferon-inducible *Mx1-Cre* transgenic mice were crossed with mice harboring loxP sequences flanking *Tal1* locus ($SCL^{\Delta/loxP}$). However, in the absence of a cell- and stage-specific promoter to drive Cre expression in macrophage precursors, an *ex vivo* approach of gene deletion had to be utilized. Mice with *loxP* sequences flanking *Tal1* locus ($SCL^{loxP/loxP}$)

(Hall et al., 2003) and another harboring the *LacZ* gene knocked into the *Tal1* locus in one allele ($SCL^{LacZ/WT}$) (Elefanty et al., 1998) have been previously described. These mice were intercrossed to generate $SCL^{loxP/LacZ}$ mice, which were identified by PCR amplification of genomic DNA extracted from tail biopsies using primers detailed in Materials and Methods (Fig. 6A). Mice with *loxP* and *LacZ* sequences gave PCR products of 304 bp and 400 bp, respectively, while mice with wild-type alleles gave a 260 bp product (Fig. 6A).

MM precursor cells were isolated from the BM of $SCL^{loxP/LacZ}$ mice, using Stanley and colleague's methods (Tushinski et al., 1982; Guilbert and Stanley, 1986; Stanley, 1990) and retrovirally transduced with MSCV-GFP-Cre vector or empty vector control. Cre expression using these vectors was tested by western blot analysis of whole cell lysates prepared from transfected NIH3T3 cells using an antibody to Cre (Fig. 6B). Cells positive for GFP fluorescence (Fig. 6C, i & ii) were sorted, put back into culture and used for subsequent analysis.

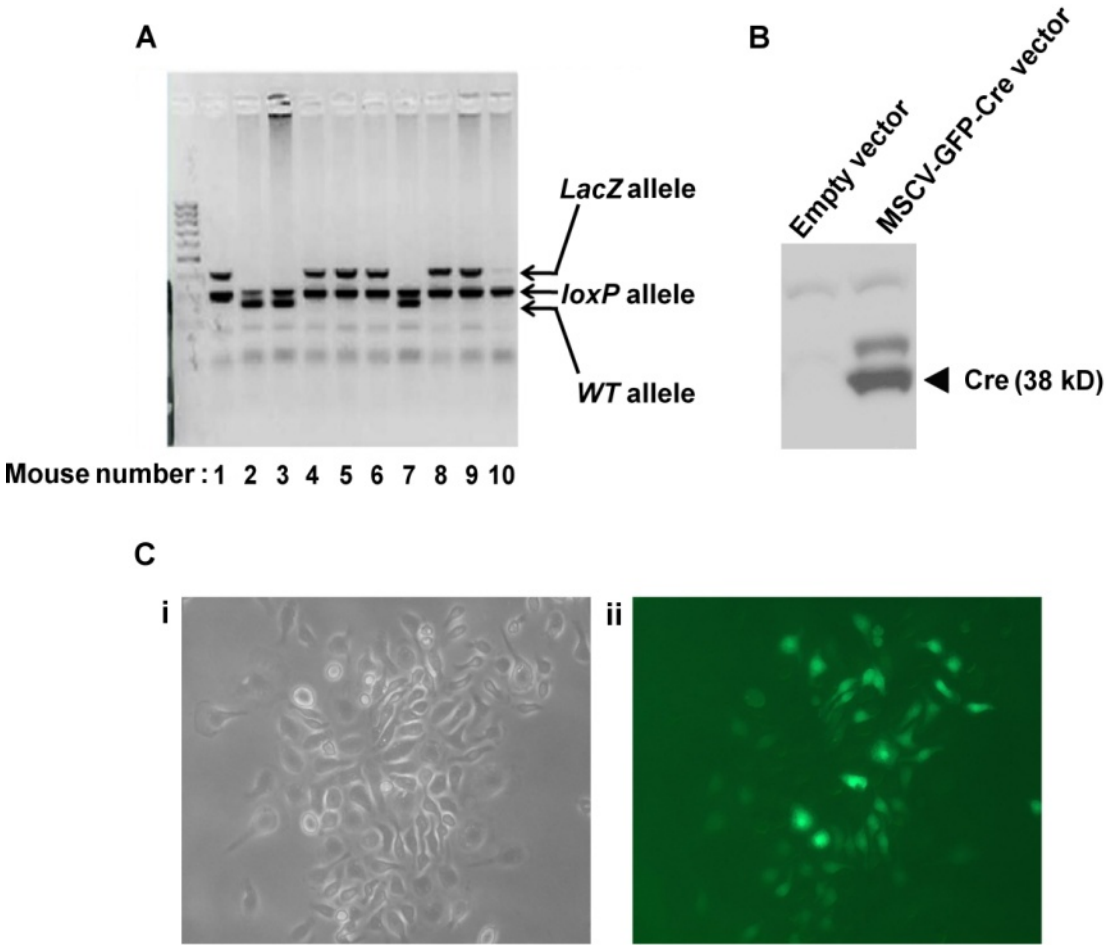

Figure 6. Genotyping of mouse genomic DNA and Cre expression by retroviral vector. **(A)** Mouse genotyping by PCR amplification of tail genomic DNA using primer pairs as described in Materials and Methods section. Mice harboring one floxed *Tal1* allele and one *LacZ* allele (as shown in lanes 1, 4, 5, 6, 8 and 9) were used for *Tal1* knockout studies. **(B)** Western blot analysis of Cre expression in whole cell lysates prepared from NIH3T3 cells transfected with either empty vector or MSCV-GFP-Cre vector. **(C)** MM precursor cells transduced with GFP-expressing vectors, viewed under visible light **(i)** or fluorescent light **(ii)** at 20× magnification.

Results

A. Efficiency of Cre-mediated deletion of Tal1 gene in MM precursor cells

BM MM precursor cells from *SCL*$^{loxP/LacZ}$ mice transduced with either MSCV-GFP-Cre vector or empty vector were sorted on the basis of green fluorescence. Cells were put into culture under identical conditions and equal starting cell numbers and induced to differentiate in presence of M-CSF (Tushinski et al., 1982; Guilbert and Stanley, 1986). The efficiency of Cre-mediated *Tal1* deletion was tested by amplification of genomic DNA from day 8 culture cells using primers specific for the excised and intact loci (Fig. 7A) (as described in Materials and Methods). Semi-quantitative PCR analysis showed a preponderance of excised loci in day 8 cells and a much smaller number with an intact locus, at this time point in culture (Fig. 7A). Presence of cells with intact locus could be a result of less than 100% efficiency in Cre-mediated deletion or because of contaminating pseudo-selected cells that may have been incorporated during sorting. Another reason could be the competitive growth advantage of *Tal1*-containing (as described later) compared to *Tal1*-null cells. In any case, RT-PCR analysis showed almost complete abolition of *Tal1* expression at all time points studied in targeted compared to vector-transduced cells (Fig. 7B). Thus, if anything, the presence of cells with the *Tal1* locus still intact (Fig. 7A) might have underestimated the extent of the *Tal1* knockout (Fig. 7B).

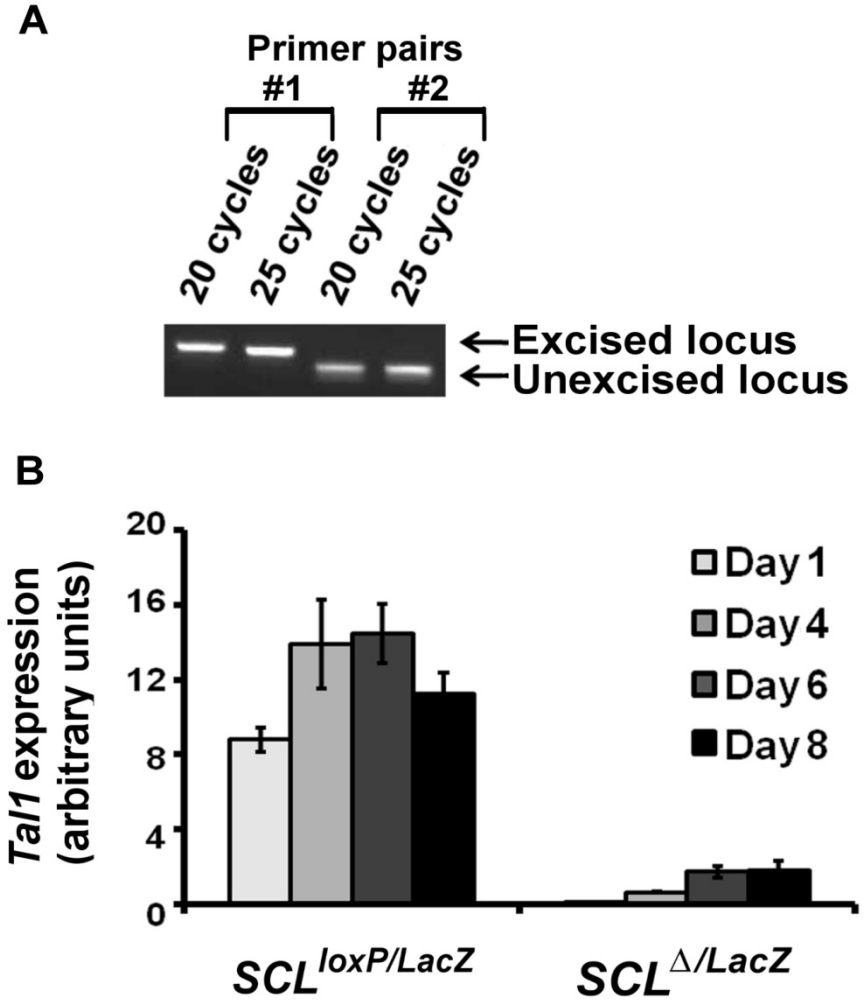

Figure 7. Efficiency of Cre-mediated *Tal1* gene deletion **(A)** Semi-quantitative PCR analysis of genomic DNA from MM precursors isolated from $SCL^{loxP/LacZ}$ mice and transduced with MSCV-GFP-Cre, cultured for 8 days after sorting. Primers described in Materials and Methods section were used to differentiate excised and intact *Tal1* locus. **(B)** Real-time PCR analysis of *Tal1* mRNA abundance in MM precursor cells isolated from $SCL^{loxP/LacZ}$ mice and transduced with parental MSCV-GFP vector ($SCL^{loxP/LacZ}$) or MSCV-GFP-Cre vector ($SCL^{\Delta/LacZ}$). Total RNA was prepared at the indicated times. Each bar represents mean ± SD from 3 independent PCR reactions as normalized to *RPS16* mRNA expression and is representative of six independent experiments.

B. Tal1 gene knockout impairs proliferation in MM precursor cells

Cell counts at timed intervals in culture showed a severe proliferative defect in *Tal1* knockout ($SCL^{\Delta/LacZ}$) compared to vector-transduced cells ($SCL^{loxP/LacZ}$) (Fig. 8A). While cells infected with the control vector increased in number by three-fold during the culture period, Cre-transduced cells showed little or no change. Toxic effects of Cre expression (Fig. 8B) or retroviral infection (data not shown) were ruled out by transduction of wild-type cells with a *Cre* expression plasmid or empty vector, respectively, with both populations found to proliferate at similar rates to non-transduced cells. In comparison, *Tal1* over-expressing cells showed a higher proliferative capacity compared to wild-type cells (Fig. 8C). Finally, in three independent experiments, heterozygous knockout cells accumulated in slightly lower numbers than wild-type cells, although this was not apparent until later times in culture (days 6 and 8) (Fig. 8C and 8D). Thus, loss of *Tal1* gene expression in differentiating MM precursors significantly impaired cellular proliferation, with *Tal1* gene expression correlating closely with proliferative potential.

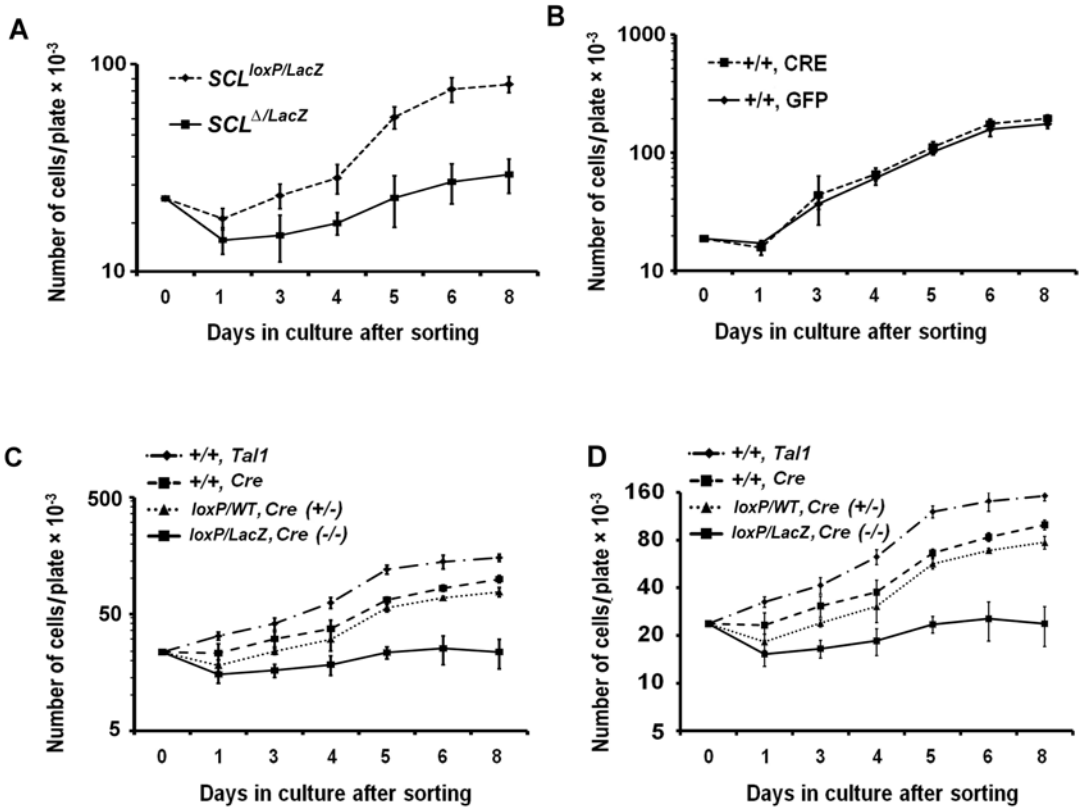

Figure 8. Effect of increase or loss of *Tal1* expression on proliferation of MM precursors *in vitro*. Retrovirally transduced GFP-expressing cells were sorted and cultured under identical conditions at equivalent initial cell concentrations. Number of cells per plate over a 8 day culture period for $SCL^{loxP/LacZ}$ *and* $SCL^{\Delta/LacZ}$ MM precursors **(A)**, wild-type MM precursors transduced with MSCV-GFP or MSCV-GFP-Cre **(B)**, and wild-type MM precursors transduced with MSCV-GFP-Tal1 or MSCV-GFP-Cre, $SCL^{loxP/WT}$ MM precursors transduced with MSCV-GFP-Cre, and $SCL^{loxP/LacZ}$ MM precursors transduced with MSCV-GFP-Cre in exponential scale **(C)** and linear scale **(D)**. Each data point represents mean ± SD from triplicate determinations, with similar trends noted in three independent experiments.

To quantify this proliferative defect in $SCL^{\Delta/LacZ}$ cells in a more accurate fashion, a flow-cytometric approach was used to measure rate of cell division in $SCL^{\Delta/LacZ}$ cells. Before sorting the cells on the basis of GFP expression, cells were stained with a membrane-intercalating, red fluorescent dye, PKH26, and only those cells that were positive for both GFP and PKH26 fluorescence were sorted and put into culture. Initial PKH26 staining was also analyzed at that time point and >98% of cells were modeled into a single parent generation (first generation, Fig. 9A i and iv). In culture, a faster rate of cell division would result in a faster decrease in PKH26 fluorescence as measured at subsequent time points by flow cytometry. Both $SCL^{loxP/LacZ}$ and $SCL^{\Delta/LacZ}$ cells were cultured under identical conditions and at equal starting cell numbers and were examined at days 6 and 8 for loss of PKH26 fluorescence from cell division. Loss of PKH26 fluorescence was analyzed and a model describing 3 or more generations of cells over the 6-8 day culture period best fit the data. Theoretically, three generations of cells should correspond to a four-fold $[2^{(3-1)}]$ increase in cell number relative to the first generation. Thus, the increase in numbers of control $SCL^{loxP/LacZ}$ cells (Fig. 8A) was only slightly less than that predicted from dye dilution studies. After 6 days, 34.8% of $SCL^{\Delta/LacZ}$ cells were still first generation, 46.8% were second generation, and only 17.8% were classified as third generation (Fig. 9A, v). Over the same time period, 13.8% of control $SCL^{loxP/LacZ}$ cells were first generation, 21.1% second generation, and 62.8% third generation (Fig. 9A, ii). While slightly less than half of $SCL^{\Delta/LacZ}$ cells (48.9%) were second generation after 8 days in culture, most $SCL^{loxP/LacZ}$ cells were in third generation, with some fourth generation cells (6.5%) also represented (Fig. 9A, iii and vi).

PKH26 fluorescence during cell culture was also measured as mean fluorescence of the population and showed a faster decrease in control $SCL^{loxP/LacZ}$ cells compared to $SCL^{\Delta/LacZ}$ cells (Fig. 9B). The control cells showed a mean fluorescence of 307.01 at day 1, which decreased to 55.53 at day 4, 49.98 at day 6 and finally to 34.28 on day 8. On the other hand, $SCL^{\Delta/LacZ}$ cells had an initial fluorescence of 191.59 (day 1) which declined to 68.19 (day 4), 67.68 (day 6) and finally to 53.89 (day 8) (Fig. 9B). Therefore, while PKH26 fluorescence for control cells decreased by 88.83% over the culture period that for $SCL^{\Delta/LacZ}$ cells showed 71.9% decrease under similar conditions.

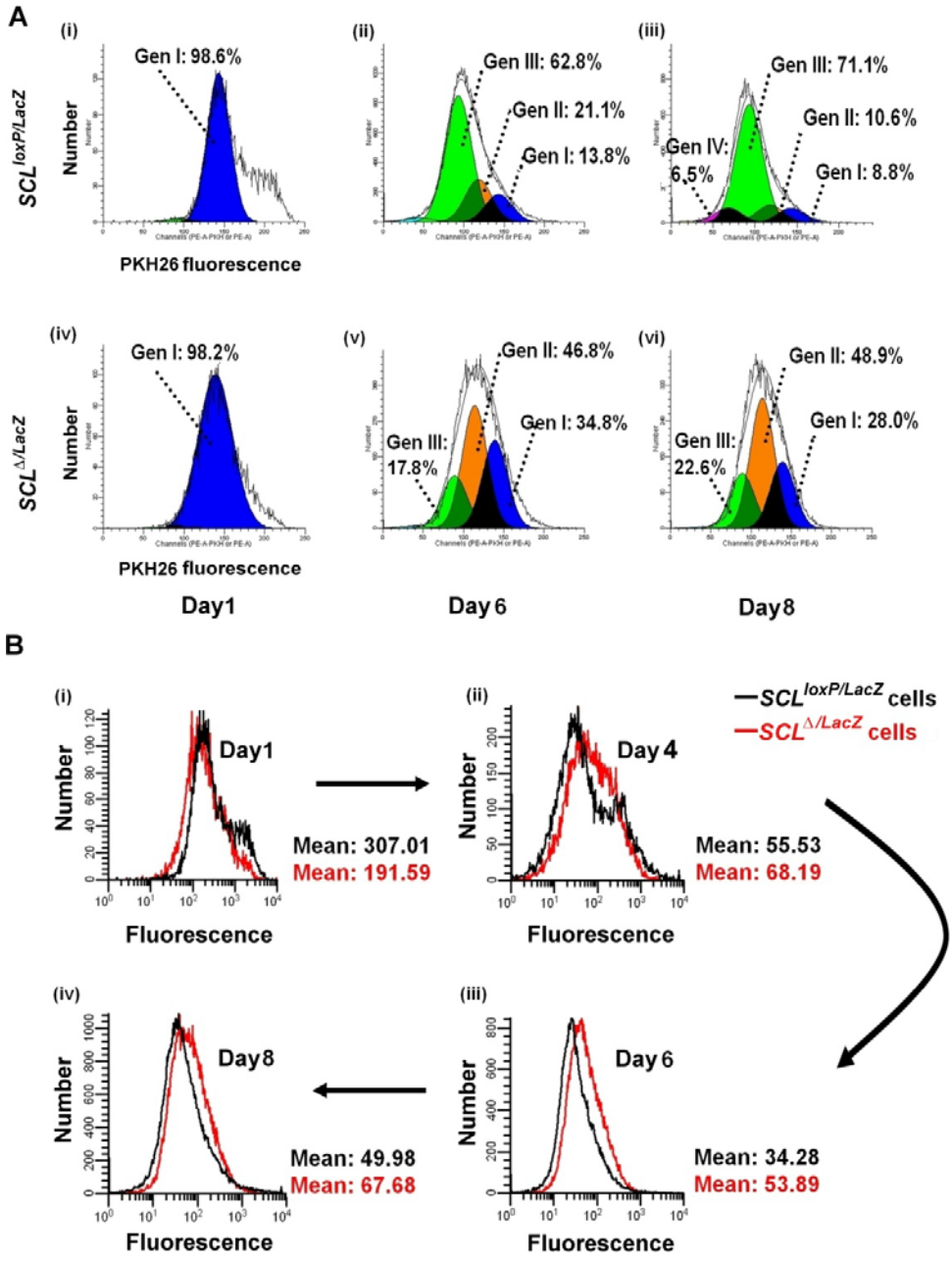

Figure 9. Proliferation analysis by PKH26 staining **(A)** $GFP^{+}PKH26^{+}$ cells were isolated using dual-color FACS and placed in culture under identical conditions at equivalent initial concentrations. Day 1 cells were modeled to represent generation I, and loss of PKH26 fluorescence intensity due to cell division, measured at days 6 and 8, were modeled as shown. The data depicted are representative of three independent experiments. Gen, generations. (B) Comparison of PKH26 dye dilution in $SCL^{loxP/LacZ}$ (black) and $SCL^{\Delta/LacZ}$ (red) cells plotted according to PKH26 incorporation. Mean fluorescence is shown.

C. Tal1 gene knockout results in modest increase in apoptosis

An early hallmark of apoptotic cells is breakup of phospholipid asymmetry of plasma membrane and exposure of phosphatidylserine (PS) residues which are translocated to the outer layer of the membrane. Annexin V preferentially binds exposed PS groups in the cell membrane and is widely used to detect early apoptotic events. APC-Annexin V staining of cells enables identification and quantification of apoptotic cells on a single-cell basis using flow cytometry. Bivariate analysis of cells stained with APC-Annexin V and 7-AAD (which binds DNA) can discriminate intact cells (APC^{neg}7-AAD^{neg}) from early apoptotic (APC^{pos}7-AAD^{neg}) and late apoptotic or necrotic cells (APC^{pos}7-AAD^{pos}). To determine whether the reduced accumulation of $SCL^{\Delta/LacZ}$ cells was attributable to decreased viability, apoptosis was analyzed using Annexin V and 7-AAD staining, with non-apoptotic cells characterized by their ability to exclude both stains (Fig. 10). The percentage of viable cells in the $SCL^{\Delta/LacZ}$ group was very slightly decreased compared to $SCL^{loxP/LacZ}$ cells at days 4 and 6 but was not different at days 1 and 8 of culture. These results showed that *Tal1* gene loss had minimal effects on cell survival and that apoptosis did not account for the marked reduction in cell number observed.

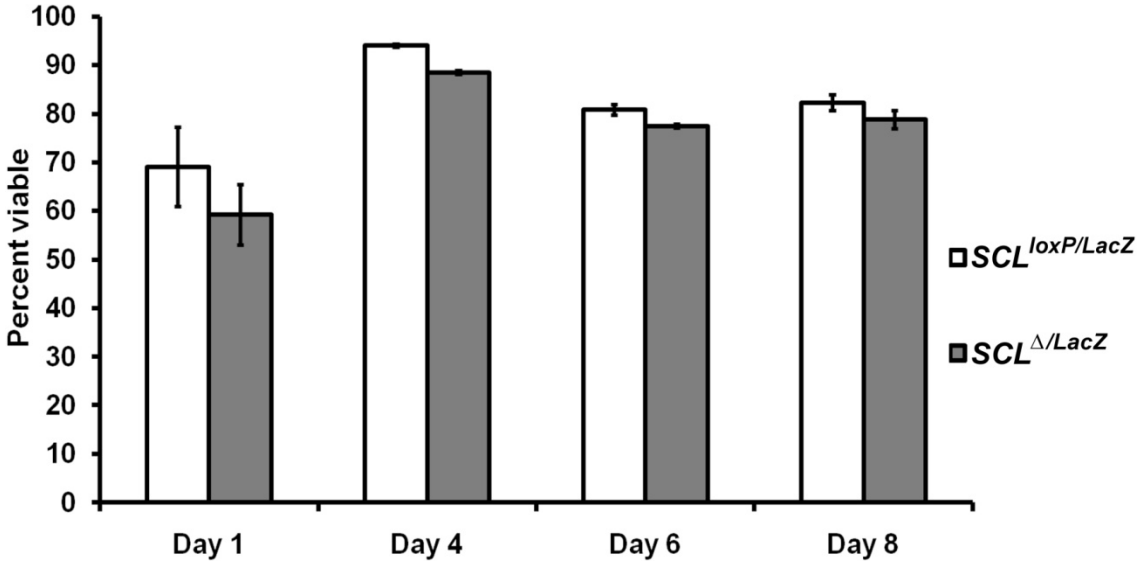

Figure 10. Apoptosis analysis of *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ MM precursor cells. Viability was determined by flow cytometry analysis of cells and defined as negative for APC-Annexin V binding and 7-AAD staining. Three plates of cells per group were analyzed at each of the indicated time points and each bar represents mean percentage ± SD of cells negative for both markers.

D. Tal1 gene knockout results in slight acceleration in differentiation

Differentiation of MM precursor cells can be tracked by expression of cell surface markers CD31 and Ly6C (Fig. 3D) (Tagoh et al., 2002). Flow cytometry analysis was used to assess expression of these two markers and to determine presence of most mature cells (of phenotype $CD31^{-}Ly6C^{-}$) in $SCL^{loxP/LacZ}$ and $SCL^{\Delta/LacZ}$ groups at three different time points in culture (Fig. 11A). Higher percentages of the most mature $CD31^{-}Ly6C^{-}$ cells were noted in $SCL^{\Delta/LacZ}$ (59.2% at day 2 and 91.2% at day 4) compared to $SCL^{loxP/LacZ}$ populations (52.2% at day 2 and 82.6% at day 4) at early times in culture. Moreover, the less differentiated $CD31^{-}Ly6C^{+}$ cells comprised a lower percentage of $SCL^{\Delta/LacZ}$ cultures (26.9% at day 2 and 8.5% at day 4) than of $SCL^{loxP/LacZ}$ cultures (36.1% at day 2 and 16.4% at day 4).

Another macrophage-specific cell surface marker, F4/80, is expressed in more mature cells but not in earlier precursor cells (Hume and Gordon, 1983). However, flow cytometry analysis of F4/80 expression did not reliably discriminate between different stages of monocytopoiesis in our hands (Fig. 11B). Approximately 90% of cells in both groups became F4/80-positive after 24 hr post-sorting and increased by almost equal amounts over the next 24 hr. In sum, these data indicate that $SCL^{\Delta/LacZ}$ cells lacking a functional *Tal1* gene differentiated somewhat more rapidly than $SCL^{loxP/LacZ}$ cells.

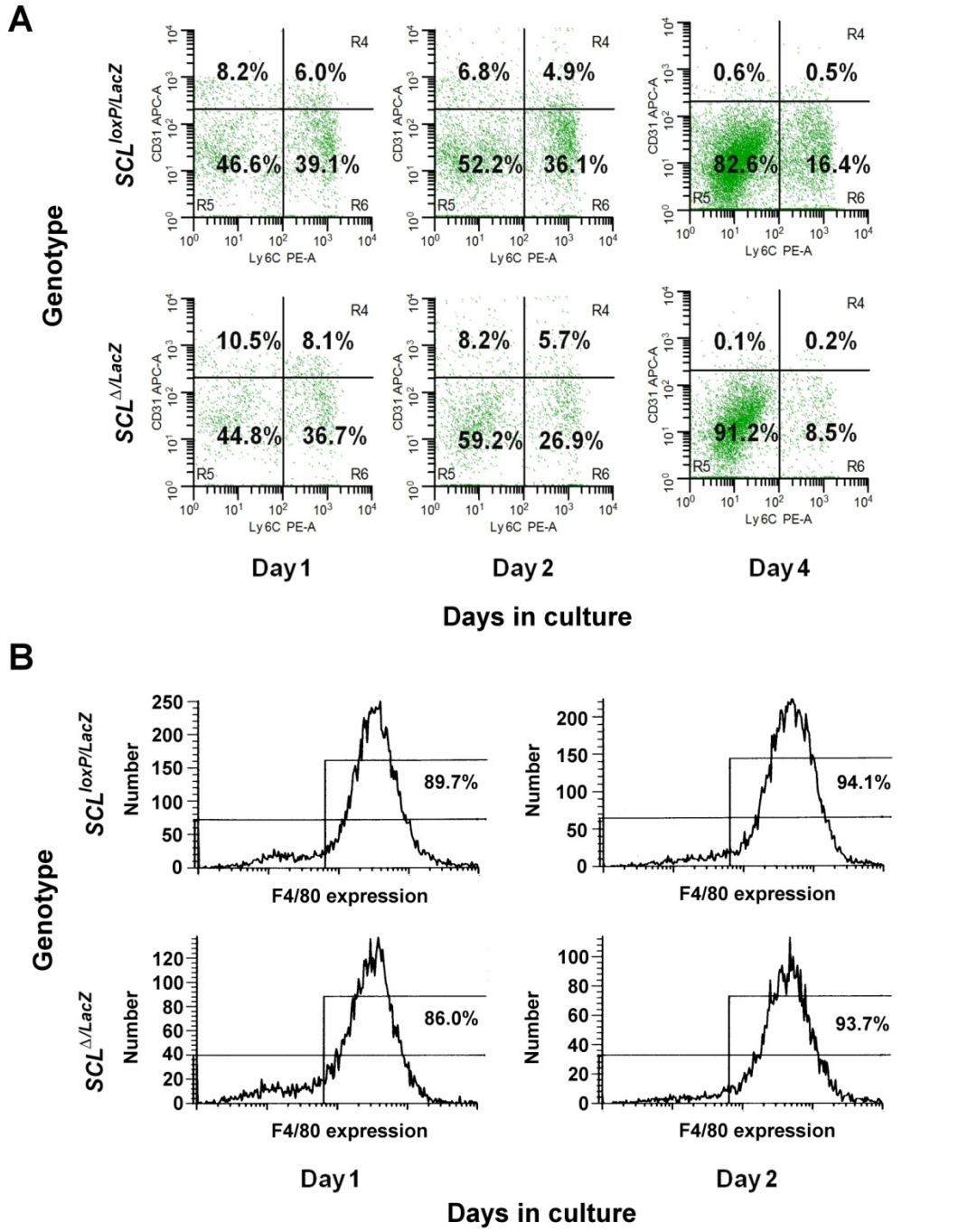

Figure 11. Analysis of differentiation of *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ MM precursor cells **(A)** Flow cytometry analysis of APC-CD31 (Y-axis) and PE-Ly6C (X-axis) staining of *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ cells at days 1, 2, and 4 of culture. Representative profile from three independent experiments is shown. **(B)** Flow cytometry analysis of F4/80 expression (X-axis) in *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ cells at days 1 and 2 of culture. Percentage of cells staining positive for APC-F4/80 mAb are denoted.

E. Tal1 knockout cells are defective in cell cycle progression

SCL$^{\Delta/LacZ}$ cells showed a significant decrease in cell proliferation which could not be explained by the modest differences in cell death and differentiation. To better characterize the proliferative defect in *Tal1* knockout cells, BrdU pulse chase analysis was carried out. The immunofluorescent staining of incorporated BrdU (an analog of the DNA precursor thymidine) and flow cytometric analysis provide a high resolution technique to determine the frequency and nature of individual cells that have synthesized DNA. To that end, GFP-expressing cells were isolated, cultured for 3 days, pulse labeled with BrdU for 45 min, and then followed through at least one cell cycle by flow cytometry. In this method, BrdU is incorporated into newly synthesized DNA by cells entering and progressing through S phase of the cell cycle during that 45 min. The cells that have incorporated BrdU can then be followed by flow cytometry through different phases of the cell cycle by staining with APC conjugated anti-BrdU antibody and 7-AAD binding of total DNA.

Analyzed immediately after pulsing, as expected, the majority of BrdU-labeled cells from both the *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ groups were in S phase (94.9% and 87.3%, respectively, Fig. 12A, i and v). At later times, *SCL*$^{loxP/LacZ}$ cells in S phase progressed rapidly to G2/M and then to G0/G1, with a corresponding decrease in the percent in S-phase (Fig. 12A ii, iii and iv). However, while some *SCL*$^{\Delta/LacZ}$ cells advanced to late S phase one hr after pulsing, as evidenced by the two peaks of labeling (Fig. 12A, vi), this population showed a higher proportion in G0/G1 after 2 and 4 hr (18.1% and 35.2%, respectively), lacked a distinct G2/M population (0% and 18.8%, respectively), and contained a higher percentage of cells still in S phase (81.9% and 46.0%) compared to

control cells (Fig. 12A, vii and viii). These results provide evidence of a slowing in cell cycle progression, with delayed traversal of S phase.

As pulse-chase approach permitted analysis of only those cells able to incorporate BrdU during the 45 min pulse, MM precursors were also cultured with BrdU for longer times (12, 24, 36, and 48 hr) before analysis by flow cytometry (Fig. 12B). Prolonged exposure of cells to BrdU allows for the comprehensive identification and analysis of actively cycling, as opposed to non-cycling, cell populations. The $SCL^{\Delta/LacZ}$ population contained a higher proportion (27.5% *vs.* 7.6% for $SCL^{loxP/LacZ}$ cells, Fig. 12B, iv and viii) of cells that never incorporated BrdU over the 48 hr labeling period.

Finally, to differentiate G0 and G1 population in both groups, flow cytometric analysis of cellular DNA and RNA content were done by staining with Hoechst 33342 and Pyronin Y dyes respectively. Cells in G0 have lower amounts of RNA and therefore lower Pyronin Y staining, while cells in G1 phase show greater staining due to their higher RNA content. Analysis of DNA/RNA staining showed a three-fold higher percentage of a quiescent or G0 population in $SCL^{\Delta/LacZ}$ cells (12.7%) compared to control $SCL^{loxP/LacZ}$ cells (4.6%) (Fig. 13). Taken together, these studies suggest a role for TAL1 in cell cycle progression in MM precursors that largely explains the proliferative defect associated with homozygous *Tal1* gene loss.

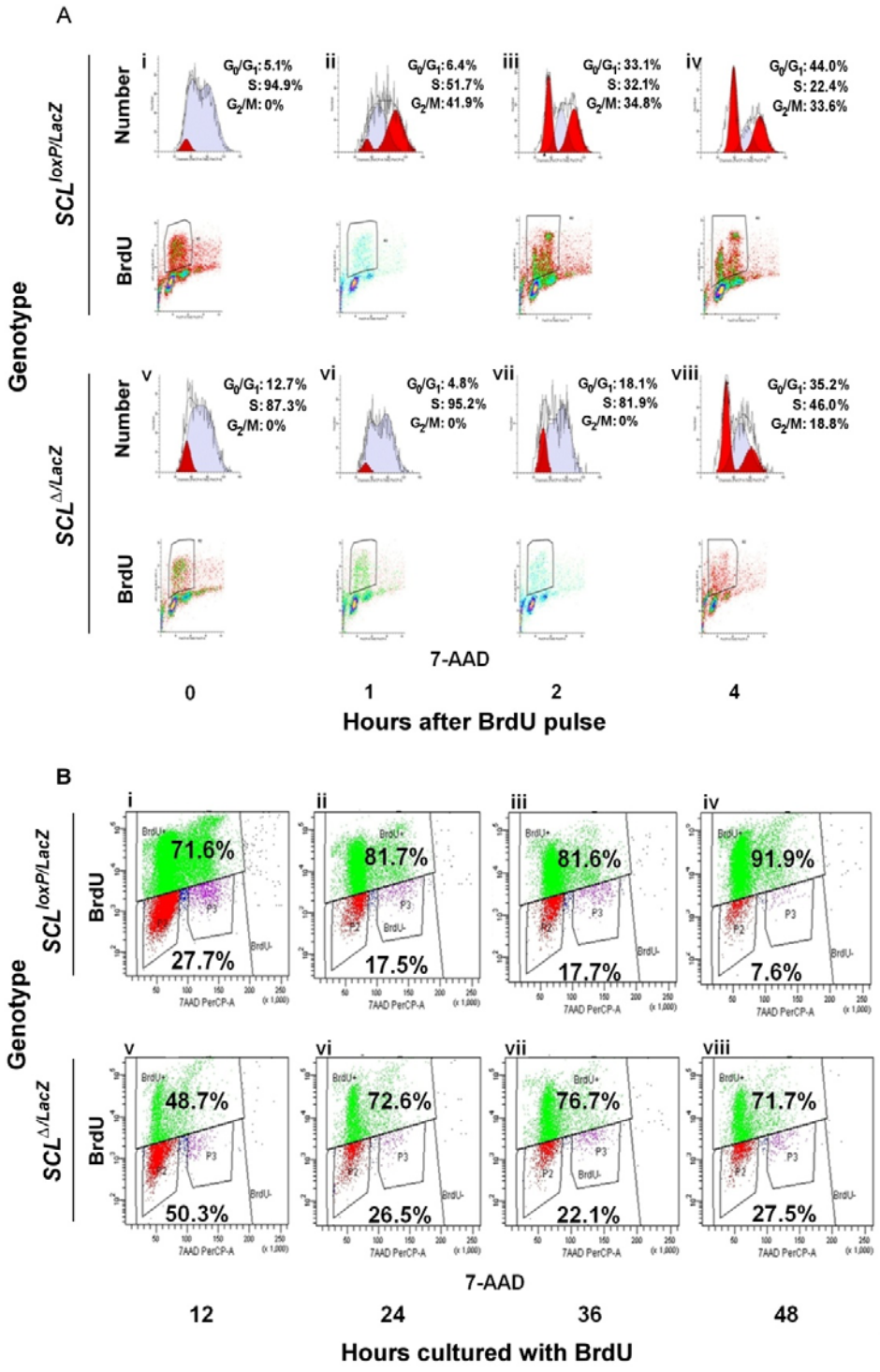

Figure 12. Cell cycle analysis of *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ MM precursor cells. **(A)** Flow cytometry analysis of BrdU incorporation *vs.* DNA content by 7-AAD staining for *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ cells at 0, 1, 2, and 4 hr after pulsing. G0/G1, S, and G2/M

populations were modeled computationally. A representative profile from multiple independent experiments is shown. **(B)** Flow cytometry analysis of *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ cells cultured with BrdU for 12, 24, 36, and 48 hr. DNA content (X-axis) is plotted *vs.* BrdU incorporation (Y-axis). Numbers inside the upper and lower quadrants denote the percentage of BrdU-positive and -negative cells, respectively.

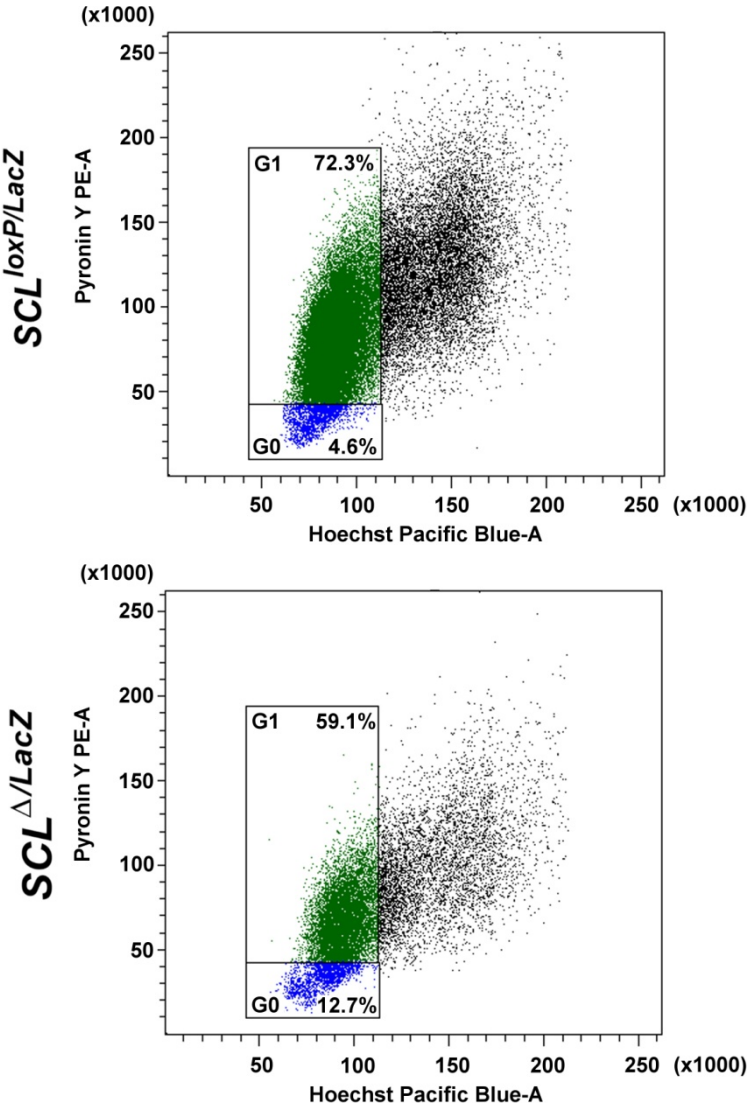

Figure 13. DNA/RNA content analysis of *SCL*$^{loxP/LacZ}$ and *SCL*$^{\Delta/LacZ}$ cells. Cells were cultured for three days after sorting of GFP-positive cells. DNA was stained with Hoechst 33342 and RNA was stained with Pyronin Y and analyzed by flow cytometry to identify Hoechst33342^{low}Pyronin Y^{low} (G0) population or Hoechst33342^{low}Pyronin Y^{high} (G1) population.

F. Gene expression analysis in Tal1 over-expressing and Tal knockout cells

Tal1, a bHLH transcription factor, could regulate downstream target genes in MM precursor cells. Identification of those target genes could explain the phenotype and also illuminate the functions of Tal1 in this lineage. To that end, gene expression analysis was carried out with wild-type, *Tal1* over-expressing and in *Tal1* knockout cells at different times in culture. Since alteration in Tal1 expression affected cell cycle progression and proliferation of these cells, the expression of several candidate genes encoding transcription factors and cell cycle regulators was tested.

By real-time PCR analysis, mRNA expression was quantified in $Tal1^{+/+}$, *Tal1* over-expressing and *Tal1* knockout ($SCL^{\Delta/LacZ}$) cells at days 1, 4, 6 and 8 in culture (Fig. 14). The same bicistronic retroviral vector used to deliver Cre to MM precursors to inactivate the floxed *Tal1* allele mice was also employed in over-expressing *Tal1* in wild-type cells. As expected, expression of *Tal1* gene was highly up-regulated in Tal1 over-expressing cells and considerably abrogated in $SCL^{\Delta/LacZ}$ cells compared to wild-type controls (Fig. 14A). Of particular significance, *Gata2* mRNA was reduced seven- and four-fold, respectively, at days 1 and 4 in $Tal1^{-/-}$ ($SCL^{\Delta/LacZ}$) relative to $Tal1^{+/+}$ cells (Fig. 14B). Although the level of *Gata2* expression decreases physiologically during differentiation of MM precursors [Fig. 3C and (Tagoh et al., 2002)], $Tal1^{-/-}$ ($SCL^{\Delta/LacZ}$) cells showed even lower *Gata2* expression than $Tal1^{+/+}$ controls at days 6 and 8. In contrast, enforced *Tal1* expression did not increase the abundance of *Gata2* mRNA at any time examined (Fig. 14B). Genes encoding other transcription factors, previously identified to be critical for monocytopoiesis, including PU.1, Runx1, Lmo2, Lmo4,

C/EBPα and C/EBPβ, were not significantly affected by the change in expression of Tal1.

Because of the delay and reduction in cell cycle progression observed with *Tal1* gene loss (Fig. 12 and 13) and the published evidence for their regulation by E proteins, the genes for several cyclin-dependent kinase (CDK) inhibitors were investigated. *p16(Ink4a)*, which is a target of repression by Tal1 (Park and Sun, 1998; Hansson et al., 2003; O'Neil et al., 2004), was up-regulated approximately 3-4 fold in $SCL^{\Delta/LacZ}$ cells and reduced, albeit to a lesser extent, in *Tal1* over-expressing cells (Fig. 14C). In contrast, expression of *p21(Cip1)*, another putative target of Tal1 and E protein regulation (Prabhu et al., 1997; Park and Sun, 1998; Liu et al., 2004), was only slightly increased in $SCL^{\Delta/LacZ}$ compared to $SCL^{loxP/LacZ}$ cells (days 6 and 8, Fig. 15A). While elevation in *p16(Ink4a)* and *p21(Cip1)* could have contributed to the cell cycle delay observed in *Tal1* knockout cells, especially given the phase of the cell cycle affected, the only slight reduction in *p16(Ink4a)* mRNA cannot explain the increased proliferation of *Tal1* over-expressing cells (Fig. 8C).

Expression of the gene encoding the transmembrane receptor, *Csf1r* was also found to be slightly up-regulated in $SCL^{\Delta/LacZ}$ cells at later time periods (days 4, 6 and 8 in culture) compared to control (Fig. 15B). This could relate to the slightly enhanced rate of differentiation noted in *Tal1* knockout cells (Fig. 11A). Finally, Tal1 over-expression up-regulated both *Csf1r* and *Il6-r* mRNA expression (Fig. 4A) and also pathways related to cell signaling and overall growth and proliferation (Fig. 5), which could have together contributed to the high proliferative potential in these cells.

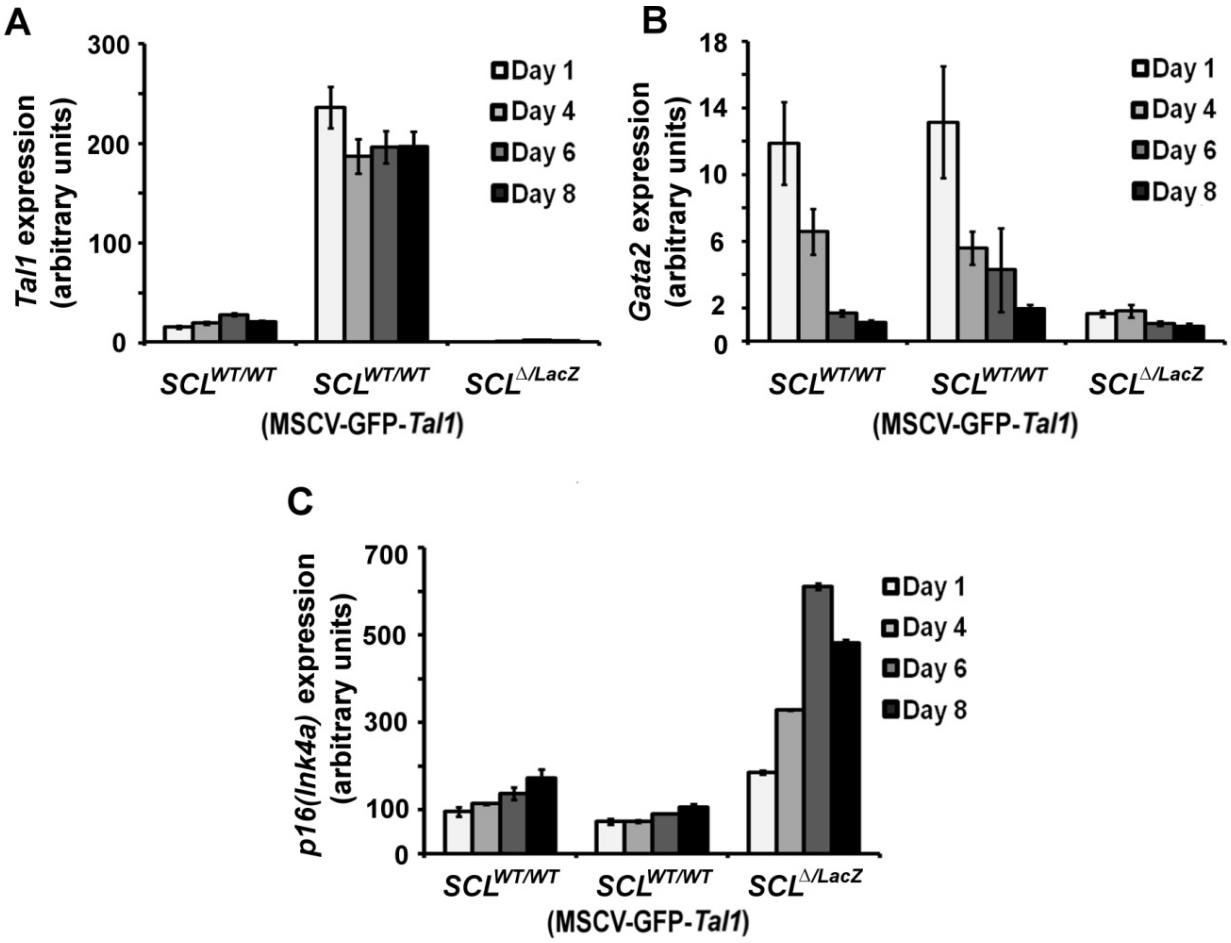

Figure 14. *Tal1* **(A)**, *Gata2* **(B)**, and *p16(Ink4a)* **(C)** gene expression analysis. Wild-type MM precursor cells were transduced with *Cre* or *Tal1* cDNA and in $SCL^{loxP/LacZ}$ MM precursor cells transduced with *Cre* cDNA ($SCL^{\Delta/LacZ}$). Cells were grown in culture for the indicated times after sorting and transcript abundance measured by real-time RT-PCR analysis. PCR reactions were done in triplicate and expressed in arbitrary units relative to *RPS16* mRNA expression. Expression profile shown is representative of three independent experiments.

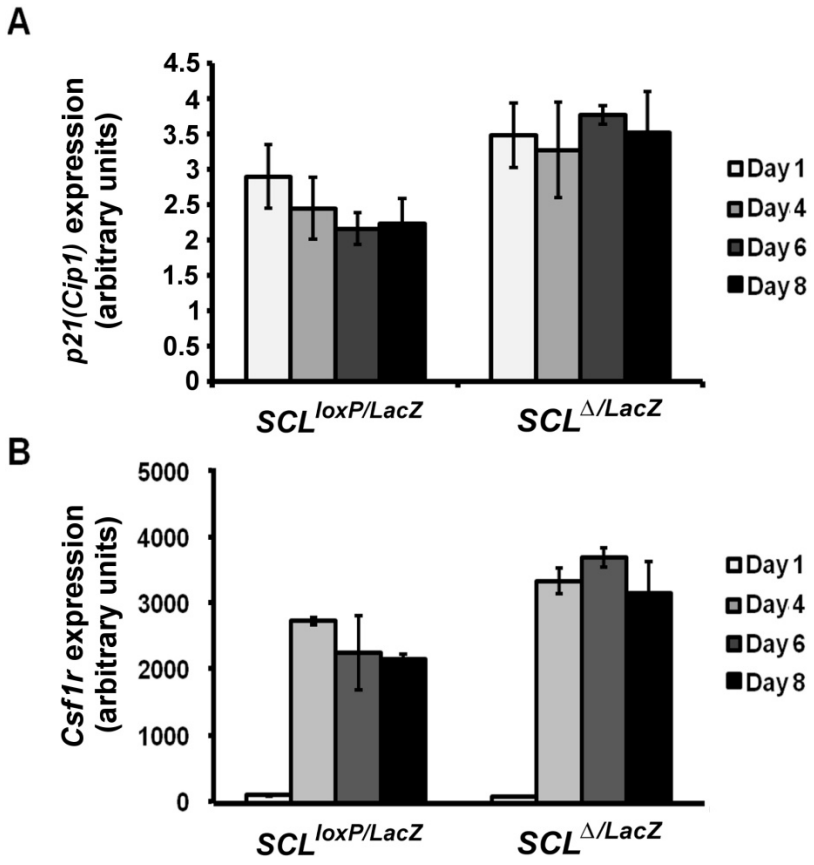

Figure 15. Expression of *p21(Cip1)* **(A)** and *Csf1r* **(B)** mRNA during differentiation of $SCL^{loxP/LacZ}$ and $SCL^{\Delta/LacZ}$ MM precursor cells. Cells were grown in culture for the indicated times after sorting and transcript abundance measured by real-time RT-PCR analysis. PCR reactions were done in triplicate and expressed in arbitrary units relative to *RPS16* mRNA expression. Expression profile shown is representative of two independent experiments.

G. Tal1 functions in MM precursor cells require direct DNA binding

TAL1 regulates transcription both through direct binding to DNA and as a non-DNA-binding cofactor (Wadman et al., 1997; Ono et al., 1998; Xu et al., 2003). To determine whether the proliferative defect in $SCL^{\Delta/LacZ}$ cells would be rescued by a wild-type *Tal1* cDNA, $SCL^{loxP/LacZ}$ MM precursors were transduced with the MSCV-GFP-Cre vector together with MSCV-YFP-Tal1, while cells transduced with the two parental vectors were used as a control. Equal numbers of GFP- and YFP-expressing cells were then sorted and cell numbers determined at timed intervals in culture. These studies showed that the defective proliferation of *Tal1* knockout cells was completely rescued by the *Tal1* cDNA, with the growth curve of *Tal1*-transduced $SCL^{\Delta/LacZ}$ cells virtually identical to that of control cells (Fig. 16). Since *Tal1* deletion resulted in down-regulation of *Gata2* expression, a *Gata2* cDNA was also tested in this rescue assay. However, cells transduced with the *Gata2* cDNA were nonviable after only 1 day in culture, likely reflecting an adverse effect of *Gata2* over-expression in a *Tal1*-null background (data not shown). Finally, to investigate whether DNA-binding activity was required for TAL1 function in MM proliferation, $SCL^{\Delta/LacZ}$ cells were transduced with the cDNA of a well-characterized DNA binding-defective mutant of Tal1, T192P (Huang and Brandt, 2000). Importantly, cells transduced with this cDNA behaved identically to $SCL^{\Delta/LacZ}$ cells and exhibited no recovery in proliferative capacity (Fig. 16). This result indicates that, similar to its actions in erythroid differentiation (Kassouf et al., 2008), Tal1 DNA-binding activity was absolutely required for outgrowth of murine MM precursors in explant cultures.

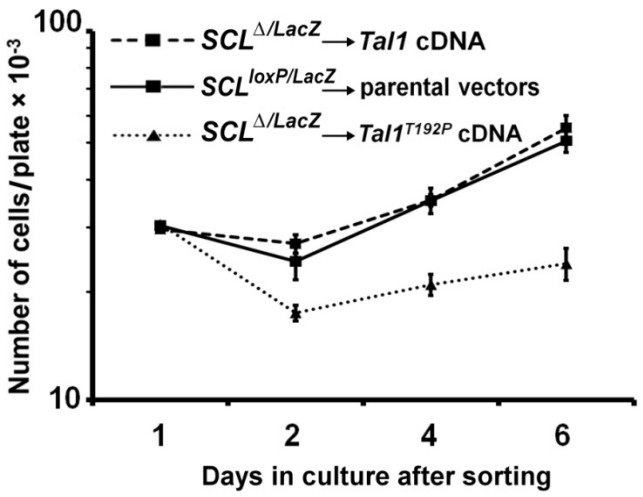

Figure 16. Rescue analysis in *Tal1* knockout cells. Cell counts in MM precursors from $SCL^{loxP/LacZ}$ mice simultaneously transduced with MSCV-GFP-Cre and either a *Tal1* or $Tal1^{T192P}$ cDNA in the retroviral vector MSCV-IRES-YFP. Dual-color FACS was used to sort GFP- and YFP-expressing cells. The $Tal1^{T192P}$ cDNA encodes a DNA binding-defective protein. Parental vector-transduced cells were used as controls, and total number of cells per plate for each day is plotted. Each data point represents mean ± SD from 3 plates of cells.

Conclusion

Tal1 gene knockout studies identified a novel function of this transcription factor in the monocyte-macrophage cell lineage. An *ex vivo* approach of gene deletion in MM precursor cells enabled the study of specific phenotypes of *Tal1* knockout and also facilitated understanding of the molecular basis of the actions. Tal1 regulated the proliferative potential of MM precursor cells in a *Tal1* dose-dependent manner, with *Tal1* over-expressing cells being highly proliferative, and even 50% reduction in Tal1 expression produced a significant difference in cell accumulation (Fig. 8C). This proliferative defect was brought about by the cell cycle regulatory effects of Tal1, as the

BrdU pulse chase analysis showed that *Tal1* knockout cells were slow in traversal through S phase. The prolonged exposure to BrdU detected higher percentage of non-cycling cells in *Tal1* knockout population at all time points and also showed relatively more cells in G0 compared to G1 phase by Pyronin Y and Hoechst 33342 staining. This suggested that a combination of slowdown in S phase traversal and a relatively higher population of non-cycling cells in $SCL^{\Delta/LacZ}$ group resulted in the proliferative defect of these cells.

Molecular gene expression analysis at different time points in culture identified two potential targets of Tal1 transcription factor, *p16(Ink4a)* and *Gata2*. While *p16(Ink4a)* gene expression was found to be up-regulated in $SCL^{\Delta/LacZ}$ cells, *Gata2* mRNA expression was reduced, suggesting a dual role for Tal1 in both up-regulating and down-regulating target gene expression. Expression of another CDK-inhibitor *p21(Cip1)*, was also found to be up-regulated, although not to the same extent as *p16(Ink4a)*, in $SCL^{\Delta/LacZ}$ cells. Interestingly, with Tal1 over-expression, *Gata2* expression did not change compared to control, and *p16(Ink4a)* expression decreased by a very small amount which cannot sufficiently explain the high proliferative potential of *Tal1* over-expressing cells. Conceivably, up-regulation of genes encoding Csf1r and Il6-r, in *Tal1* over-expressing cells resulted in increased sensitivity to growth factors and contributed to their high proliferative potential (Fig. 4).

Up-regulation of CDK-inhibitors is connected with cell cycle delay (Sherr and Roberts, 1999) and in this case, may have contributed to the phenotype observed in *Tal1*-null cells. Moreover, Gata2 regulates cell cycle progression in G1ME cells (Huang et al., 2009) and could have contributed to the delay in cell cycle progression. Decreased Gata2

expression in G1ME cells also correlated with up-regulation of myeloid gene expression (Huang et al., 2009), similar to what was seen in MM precursors in which reduced *Gata2* expression in $SCL^{\Delta/LacZ}$ cells led to increased *Csf1r* expression (Fig. 14B and 15B).

Reduction in Gata2 expression accelerates differentiation in adipocytes (Tsai et al., 2005; Okitsu et al., 2007) and monocytes in juvenile myelo-monocytic leukemia (JMML) patients (Yang et al., 2009). This could explain the enhanced differentiation seen in $SCL^{\Delta/LacZ}$ MM precursor cells at early culture periods (Fig. 11A). Interestingly, rescue attempt by re-introduction of *Gata2* cDNA in $SCL^{\Delta/LacZ}$ cells resulted in complete loss of cell viability (data not shown). Earlier studies have documented a complete loss of colony-forming ability of BM progenitor cells over-expressing *Gata2* although those cells remained completely viable. Therefore, the high rate of cell death observed in rescue of $SCL^{\Delta/LacZ}$ cells with *Gata2* could be due to an adverse effect of *Gata2* over-expression in a *Tal1*-null background.

Finally, re-introduction of a *Tal1* cDNA resulted in complete rescue of the proliferative defect of MM precursor cells, which suggests that the $SCL^{\Delta/LacZ}$ phenotype was due to loss of the *Tal1* gene directly and not due to any indirect effect. Moreover, the DNA-binding mutant of Tal1 did not exhibit recovery of proliferative capacity, indicating that, similar to its actions in erythroid differentiation (Kassouf et al., 2008), Tal1 DNA-binding activity was absolutely required for outgrowth of murine MM precursors in explant cultures.

CHAPTER VI

TAL1 DNA BINDING IN MM PRECURSOR CELLS AT GENE REGULATORY REGIONS

Introduction

Tal1 is a class II bHLH transcription factor that can heterodimerize with class I bHLH proteins called E proteins and recognize specific E box sequences (CANNTG) in gene regulatory regions. The E box-binding element preferred by this heterodimer is CAGATG, with preference extending to two bases on either side of the E box as well (Hsu et al., 1994a). A multimeric complex consisting of TAL1-E protein heterodimer bound to an E box, and bridged by Ldb1 and LMO2 to a GATA protein bound to a GATA site 9-12 bp away was also identified in MEL cells (Wadman et al., 1997; Xu et al., 2003; Xu et al., 2007). As a result of its association at gene regulatory regions with different partners, Tal1 activates and represses transcription of target genes, this being critical not only in specification of hematopoietic cell fate and vasculogenesis but also in leukemogenesis.

Tal1-E protein complexes bind DNA with a reduced transactivation potential compared to E protein homodimers (Hsu et al., 1994c; Park and Sun, 1998) and can also repress the transcription of genes with E box sequences in their promoter. This has been shown previously in reporter experiments with promoter regions of several genes including *p16(Ink4a)*, *p21(Cip1)* and *pTα* (Prabhu et al., 1997; Funato et al., 2001; Hansson et al., 2003; Liu et al., 2004). Mutational analysis of the upstream E box

sequences in promoter regions have shown that those elements are critical for full transcriptional activation by E proteins and co-expression of Tal1 resulted in repression of this activation. This suggested that Tal1 might have a direct role in regulating the expression of these genes by binding E box sequences in upstream regulatory regions. The oncogenic potential of Tal1 may also be related to this characteristic as observed in a study done with E2A- or HEB-heterozygous mice (O'Neil et al., 2004). Ectopic expression of Tal1 in thymocytes of those mice resulted in T cell differentiation arrest and accelerated leukemogenesis suggesting that Tal1 interfering with E protein function may be a critical mechanism in developing T cell malignancy (O'Neil et al., 2004).

Gata2 is expressed in a wide variety of tissues including hematopoietic, endothelial and neuronal cells and, *Gata2* gain-of-function experiments and *in vitro* differentiation assays in *Gata2*$^{-/-}$ ES cells showed that it plays a pivotal role in the proliferation of early hematopoietic progenitors (Briegel et al., 1993; Tsai and Orkin, 1997; Kitajima et al., 2002). An E box-GATA motif in an intronic enhancer region (+9.5 kb) of the *Gata2* gene is critical for its expression in endothelial and fetal liver cells during embryonic development (Khandekar et al., 2007; Wozniak et al., 2007). This single motif was indispensable and sufficient for *Gata2* expression in hematopoietic cells, whereas for expression in human endothelial cells it required additional regulatory modules (Wozniak et al., 2007). Electrophoretic mobility shift analysis (EMSA) showed that Tal1-E protein heterodimers could bind with high affinity to this crucial enhancer region *in vitro*, which could be competed with a cold wild-type probe but not one with a mutated E box (Khandekar et al., 2007). In reporter assays done in the G1ME and MEL

erythroid cell lines, this element showed robust activity, while mutation or removal of the GATA sites resulted in abrogation of erythroid enhancer activity (Grass et al., 2006).

The increase in *p16(Ink4a)* and reduction in *Gata2* mRNA expression in $SCL^{\Delta/LacZ}$ MM precursor cells suggests that Tal1 plays a role in regulating the expression of these two genes. Moreover, given the phenotypic abnormalities seen in $SCL^{\Delta/LacZ}$ cells, de-regulation of *p16(Ink4a)* and *Gata2* expression seemed to contribute to this defect. Since the DNA binding-defective mutant of Tal1 was not able to rescue the defects, it is probable that Tal1 function in this lineage is mediated by direct interaction with DNA. On that basis, we hypothesized that Tal1 regulates the expression of *p16(Ink4a)* and *Gata2* by directly occupying upstream regulatory regions in association with its E protein binding partner.

Results

To identify probable Tal1-binding sites in *p16(Ink4a)* upstream region we scanned a 12 kb region upstream of the translational start site and identified 5 preferred E box sequences (CATCTG or CAGATG) for TAL1-E protein binding (bases -13, -2592, -4638, -5106, and -6932) (Fig. 17A). The preferred E box at -4638 has two other adjacent non-canonical E boxes at -4650 and -4719, which could also participate in complex formation at this region. MM precursor cells at days 4 and 7 in culture were used for ChIP analysis with antibodies to Tal1 and E47 and control IgG. The amount of DNA immunoprecipitated was then quantified by real-time PCR analysis using specific primers flanking each of these regions or the gene's 3´ UTR and expressed as a percentage of

input. Three of these E boxes, at -2592, -4638 and -5106, were shown by this approach to be occupied by Tal1 and E47 in day 4 cells (Fig. 17C). In contrast, no binding was detected at -13, -6932, or the 3′ UTR at any time (data not shown), and another, non-canonical E box in the promoter region (-437) likewise did not show binding (data not shown). Finally, Tal1 and, unexpectedly, E47 occupancy of the -2592 and -4638 elements were very low and essentially absent at the -5106 E box at day 7 (Fig. 17D), despite the increase in *p16(Ink4a)* expression at this time (Fig. 3C and 14C). This suggests the possibility that E proteins other than E47 are involved and/or other mechanisms for *p16(Ink4a)* gene transactivation operate at these later times.

Another putative Tal1-E protein target is the *Gata2* intron 4 (+9.5 kb) enhancer region, which consists of three GATA sites and an E box element (Fig. 17B) that regulated Gata2 expression in endothelial and hematopoietic cells (Khandekar et al., 2007; Wozniak et al., 2007). To determine whether Tal1 occupied this region in MM precursors, ChIP analysis was carried out as above with antibodies to Tal1 and E47 and with IgG as a control. Tal1- and E47-specific antibodies, but not IgG, were able to precipitate this intronic fragment from day 4 (Fig. 17C) but not day 7 cells (Fig. 17D), concordant with the decline in *Gata2* expression over this same time period (Fig. 3C and 14B). Primer sequences designed for the *Gata2* 3′ UTR did not support any amplification (data not shown), indicating specific occupancy of this intronic region in MM precursor cells.

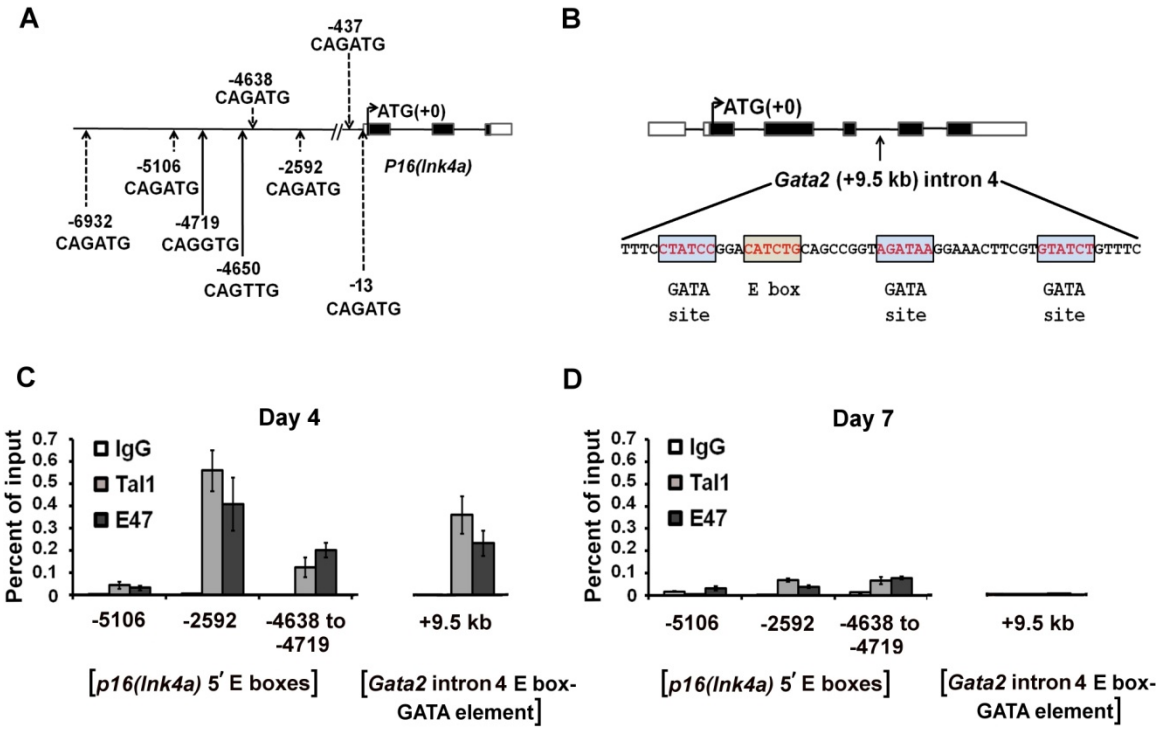

Figure 17. Chromatin immunoprecipitation analysis in MM precursor cells. Day 4 and day 7 cells were used to detect Tal1 and E47 association at *p16(Ink4a)* 5' E boxes **(A)** and *Gata2* intron 4 E box-GATA element **(B)**. Noncoding exons are denoted by empty boxes and coding exons are denoted by solid boxes. The preferred E box sequences in *p16(Ink4a)* upstream region are pointed with dashed lines and non-canonical E boxes pointed with solid lines and the 55 bp region in *Gata2* intron 4 containing the E box-GATA element is also shown. Sonicated chromatin fragments from day 4 **(C)** and day 7 **(D)** MM precursor cells, immunoprecipitated with antibodies to Tal1 or E47 or normal rabbit IgG were quantified by real-time PCR analysis using region-specific primers. Bar represents mean abundance ± SD of each fragment relative to input as determined from triplicate PCR amplifications and is representative of three independent experiments.

In murine myeloid leukemia cell line M1, p16(Ink4a) protein expression goes up with IL-6-induced differentiation (Amanullah et al., 2000) in parallel with a decrease in Tal1 expression and Tal1/E protein DNA-binding activity (Voronova and Lee, 1994). As this phenocopies the situation with *Tal1* gene knockout, ChIP analysis was also carried out on chromatin from untreated and IL-6-treated M1 cells for the same regions that were tested in primary MM precursor cells. The IgG control did not show binding for any of the regions, and the 3' UTR region did not show binding for any of the specific antibodies (Fig. 18A). As predicted from the drastic decline in its expression (Voronova and Lee, 1994), Tal1 occupancy was detected only in uninduced cells and not in cells treated with IL-6 for 48 hr (Fig. 18A and 18B). In contrast, E2A association with these same E box elements could be detected in both IL-6-treated and untreated cells, indicating that its E12 and/or E47 protein products would be unopposed by TAL1 to augment expression of this gene when these cells differentiate (Fig. 18C).

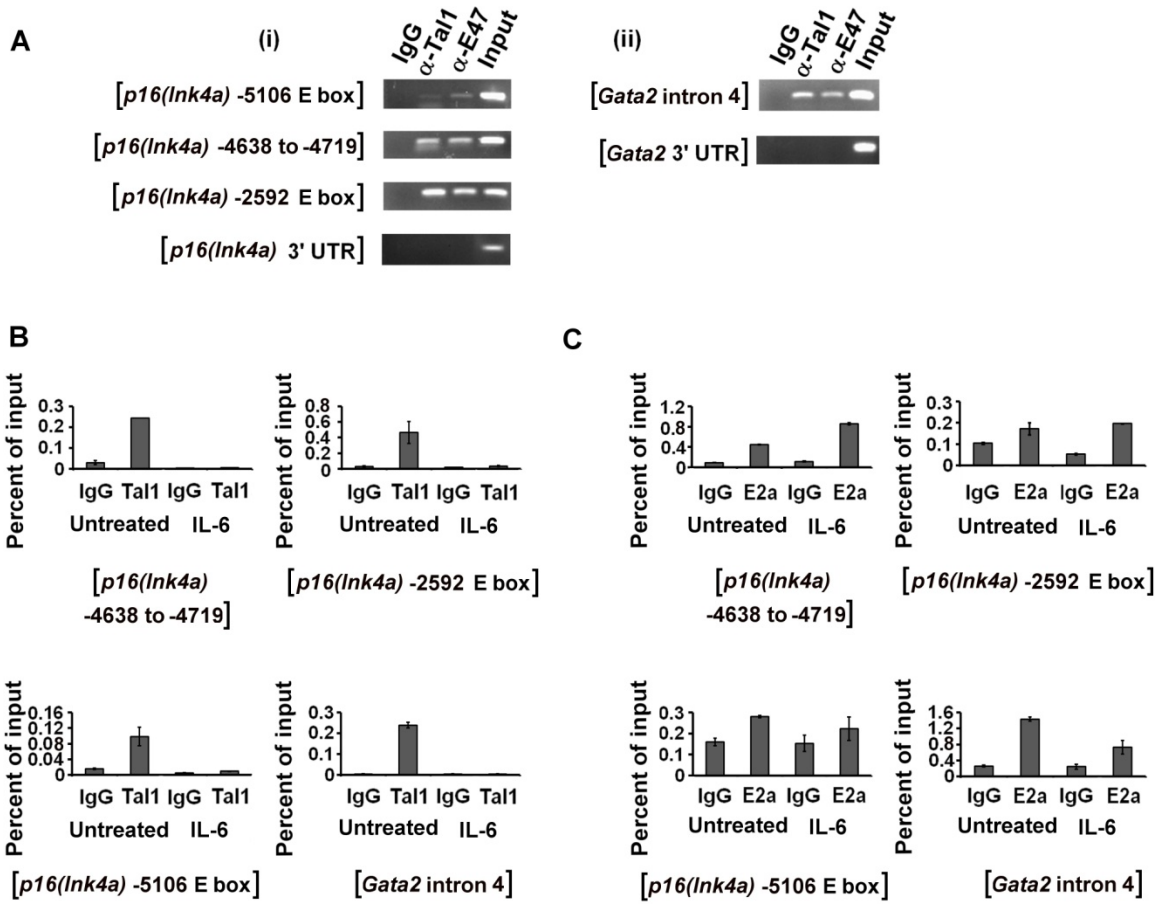

Figure 18. Chromatin immunoprecipitation analysis in M1 cells. ChIP analysis of Tal1 and E47 binding at *p16(Ink4a)* 5' E boxes **(A)(i)** and *Gata2* intron 4 **(A)(ii)** in M1 cells crosslinked with formaldehyde. Sonicated chromatin fragments were immunoprecipitated with antibodies to Tal1 (α-Tal1), E47 (α-E47), and control rabbit IgG. Quantitative PCR analysis was done with specific primers to compare Tal1 **(B)** and E2A **(C)** association, relative to input in untreated M1 cell. Some cells were treated with IL-6 for 48 hr. The results shown are representative of three independent experiments.

Conclusion

The Tal1-E protein binding studies identified a novel function of these two proteins in differentiation of MM precursor cells and demonstrated that the *p16(Ink4a)* and *Gata2* genes are direct targets of Tal1in this lineage. It had been previously reported that *p16(Ink4a)* expression could be controlled by Tal1 and E protein association at specific E boxes in its promoter region (Nielsen et al., 1996; Park and Sun, 1998; Hansson et al., 2003). However, these studies utilized promoter-reporter constructs transiently transfected into cell lines; still, they showed that mutation or deletion of these E boxes severely affected reporter gene expression. These studies also demonstrated that Tal1 is very important for regulation of gene expression, since E protein homodimers function as more potent activators of transcription than Tal1-E protein heterodimer. Our ChIP analyses carried out in primary MM precursor cells, establish that Tal1-E protein heterodimers occupy these gene regulatory regions in the appropriate cell types at the appropriate times and suggest a mechanism for Tal1 function in regulating cell proliferation and cycling. It also shows that the heterodimer associates selectively with certain E box sequences (-2592, -4638 and -5106) but not with the more proximal (-13 and -437) and the most distal (-6932) E boxes that were analyzed. Finally, the fact that Tal1-E47 association is lower at day 7 of culture compared to day 4, although *p16(Ink4a)* gene expression is higher at later times, suggests that other E proteins may also be involved with regulation of this gene or that alternative modes of regulation exist.

The Gata2 zinc finger transcription factor has a critical function in the hematopoietic system, and the *Gata2* intron 4 enhancer region was regulated by a Tal1-E protein complex in endothelial cells and hematopoietic cells (Khandekar et al., 2007;

Wozniak et al., 2007). Our findings that the same region is bound in MM precursor cells by the Tal1-E protein complex suggest an important function of Tal1 in this lineage. The absence of complex association with this intron in day 7 cells correlated with Gata2 expression in this lineage which decreases with differentiation. This could be an important event in macrophage differentiation, since ectopic expression of Gata2 in an attempt to rescue the phenotypic defect in $SCL^{\Delta/LacZ}$ cells resulted in complete loss of cell viability.

Lastly, ChIP analysis of Tal1-E protein association in the M1 leukemic cell line show findings similar to primary cells and suggests a mechanism for repression of *p16(Ink4a)* expression in uninduced cells. Differentiation-inducing signals induce p(16Ink4a) expression and loss of leukemogenicity (Amanullah et al., 2000) along with a decrease in Tal1 expression and abrogation of Tal1-E protein heterodimer (Voronova and Lee, 1994).

CHAPTER VII

DISCUSSIONS AND FUTURE DIRECTIONS

The studies described above investigate and analyze a previously unknown role of bHLH transcription factor TAL1 during monocytopoiesis. Extensive studies have been done to identify function of TAL1 in other hematopoietic lineages including erythrocytes, megakaryocytes, and mast cells. Although derived from the same progenitor cells as these, the CMPs, there is conflicting evidence of TAL1 function in MM precursor cells.

Work carried out in a human cell line TF-1, which has the dual potential of differentiating towards both erythrocytic and monocytic lineages, found that Tal1 expression decreased slightly with differentiation (Hoang et al., 1996). The same study also showed that constitutive expression of Tal1 resulted in a decrease in adherent cells, reflected in reduced immunoreactivity towards CD45 and FcγRII (CD32), which are, however, not macrophage-specific markers and do not reliably specify monocytopoiesis. Moreover, in the M1 murine myelo-monocytic leukemia cell line that can be induced to differentiate to macrophage by both leukemia inhibitory factor (LIF) and IL-6, ectopic expression of Tal1 enhanced IL-6-induced differentiation as measured by Mac1α expression but perturbed LIF-induced differentiation (Tanigawa et al., 1995). Nuclear extracts prepared from uninduced M1 cells were analyzed by EMSA using an E box oligonucleotide probe and showed presence of Tal1-E protein heterodimer bound specifically to the E box DNA motifs (Voronova and Lee, 1994). However, DNA binding of the Tal1-E protein heterodimer became undetectable after 24 hr of IL-6 treatment,

when differentiation-induced morphological and molecular changes are initiated in M1 cells (Voronova and Lee, 1994). In fact, with M1 cell differentiation, Tal1 expression is extinguished and expression of dominant-negative HLH proteins Id1, and primarily Id2, is increased, while E12 and E47 protein expression is unaffected (Voronova and Lee, 1994).

However, several groups have detected Tal1 expression in primary mouse macrophages and peripheral blood mononuclear cells and BM macrophages (Kallianpur et al., 1994) and in zebrafish (Zhang and Rodaway, 2007) and human macrophages (Pulford et al., 1995). The mouse *Tal1* cDNA was cloned from a BM macrophage cDNA library by using HLH-specific oligonucleotides as hybridization probes (Begley et al., 1991). One aspect of studies done with myeloid leukemia cell lines is that they do not realistically reproduce the physiological condition and differ not only in expression pattern of Tal1 but also in cytokines responsible for induction of differentiation. Differentiation of M1 murine monocytic leukemia cells is induced by LIF or IL-6 while differentiation of TF-1 human bipotent cells is induced by TPA. However, differentiation of primary BM mononuclear precursors towards macrophage lineage initially requires a combination of IL-3 and M-CSF and only M-CSF at later stages of differentiation.

In vivo deletion of *Tal1* in HSCs in interferon-inducible *MxI-Cre* mice perturbed megakaryopoiesis and erythropoiesis with the loss of early progenitor cells, but did not appear to alter macrophage, granulocytic, mixed granulocyte/macrophage progenitor numbers in BM and spleen and formed colonies of similar size to controls (Hall et al., 2003; Mikkola et al., 2003). However, loss of *Tal1* in cells nullizygous for the related *Lyl1* gene showed ten-fold reduction in myeloid colony numbers arising from LKS cells,

raising the possibility of genetic redundancy (Souroullas et al., 2009). Therefore, although the evidence suggests that Tal1 plays an active role in monocytopoiesis, previous studies were not definitive and could neither rule in or out Tal1 involvement in monocytopoiesis.

In the present work we aimed at studying the role of this critical transcription factor in monocytopoiesis and investigated the molecular mechanism underlying its actions in this lineage. Using two different *ex vivo* differentiation systems, one involving CMPs and another involving more committed progenitor cells consisting of monoblasts and promonocytes, we detected Tal1 expression at all stages of monocytopoiesis and even in activated macrophages. Previous studies have looked at Tal1 expression in peripheral blood mononuclear cells and tissue macrophages (Begley et al., 1989; Kallianpur et al., 1994; Pulford et al., 1995), but this is the first comprehensive study of Tal1 mRNA and protein expression during monocytopoiesis.

The over-expression studies showed that Tal1 has an active role in monocytopoiesis and confers a proliferative advantage to the cells (Fig. 8) possibly by inducing expression of cell surface growth factor receptors (Fig. 4). Two groups have investigated the effects of Tal1 over-expression in mouse BM reconstitution. One study could not detect any phenotype in mice over-expressing *Tal1* driven by the LXSN retrovirus (Elwood and Begley, 1995; Elwood et al., 1998). However, another study showed that over-expression of Tal1 promoted myeloid repopulation, which did not persist beyond 10 weeks (Kunisato et al., 2004). Interestingly, transduction of *TAL1* cDNA increased the proliferation of the 32D cells via an anti-apoptotic effect and HL-60 cells via increased DNA synthesis (Condorelli et al., 1997).

Microarray gene expression analysis in Tal1 over-expressing macrophages showed up-regulated immune response, cell signaling and proliferation pathways, (Fig. 5) which could be related to the overall augmentation of differentiation. Interestingly, expression of the DNA binding-defective mutant of Tal1 did not enhance *Csf1r* or *Il6-r* gene expression which suggests that direct interaction with DNA may be necessary to elicit these effects.

Similar findings were also seen in M1 myeloid leukemia cells (Fig. 4), and the high proliferative capacity of Tal1 over-expressing cells suggest that it might be relevant to actions of TAL1 in T-lymphoid and, conceivably, myeloid leukemias. TAL1 expression in human chronic myeloproliferative disorders has not been extensively documented and there is no evidence of chromosomal translocations or point mutations that affect the TAL1 locus in myeloid leukemia (Rockman et al., 1992; Delabesse et al., 2003). However, increased TAL1 expression was detected in 7 out of 8 cases of chronic myeloid leukemia with myeloid blast crisis (Shimamoto et al., 1995). Moreover, de novo AML patients who expressed TAL1 had poor prognosis, survived for less than 1 year after diagnosis and showed poor complete remission (C.R.) rate (Shimamoto et al., 1995; Ohyashiki et al., 1996). Comparative microarray gene expression analysis of $CD34^{+}$ cells from BM of untreated CML patients have also detected increased expression of TAL1 and GATA2 (Diaz-Blanco et al., 2007). Finally, increased TAL1 expression has also been described in peripheral blood mononuclear cells in patients with myelofibrosis with myeloid metaplasia (Steunou et al., 2003). Future studies aimed at more detailed analysis of role of TAL1 in myeloproliferative disorders, particularly in chronic

myeloproliferative diseases may explain these findings and also elucidate pathways that are altered by Tal1 over-expression in myeloid cells.

Complete loss of *Tal1* gene resulted in proliferative and cell cycle defects in macrophage precursors, and even wild-type and heterozygous knockout cells also differed in their growth rates. This was most apparent at later times in culture (days 6 and 8, Fig. 8), which suggested that more differentiated cells were most sensitive to *Tal1* gene dose. Although earlier *in vivo* gene deletion studies did not reveal any defect in macrophage, granulocytic, or mixed granulocyte/macrophage lineage (Hall et al., 2003; Mikkola et al., 2003), *ex vivo* deletion of the *Tal1* gene, as carried out here brought out phenotypic abnormalities, either because of the acute nature of gene loss or because culture conditions simulated stress monocytopoiesis. In support of the latter, $SCL^{\Delta/LacZ}$ mice showed a small but significant delay in the recovery of circulating monocytes after 5-FU treatment (unpublished data, Dr. David Curtis, The Royal Melbourne Hospital, Australia). The cell cycle studies in $SCL^{\Delta/LacZ}$ cells showed an overall decrease in rate of progression through S phase and increase in G0 population while the prolonged BrdU-labeling studies identified a higher percentage of cells that never entered cell cycle. It is possible that the non-cycling cells represented a population that had the highest efficiency in *Tal1* gene deletion and exhibited the most severe phenotype.

The proliferative defect and the particular cell cycle phases that got affected associated with *p16(Ink4a)* and *p21(Cip1)* gene up-regulation and were previously shown to be positively regulated by E proteins, while Tal1-E protein heterodimers were suggested to repress their transcription (Doyle et al., 1994; Hofmann and Cole, 1996; Prabhu et al., 1997; Park and Sun, 1998; Hansson et al., 2003; O'Neil et al., 2004). Those

studies have shown that E protein homodimers transactivate transcription by binding E box sequences in promoter regions, and Tal1-E protein heterodimer binding at the same sites repress this activation. Reporter assays have also shown that mutation of these E boxes result in complete abrogation of promoter activity and hence direct binding to these sequences is important to elicit gene expression. Moreover, in the Jurkat cell line in which Tal1-E2A transcriptional activity is low, restoration of E2A activity profoundly suppresses growth and increases apoptosis (Park et al., 1999). Thus, in *Tal1* knockout MM precursor cells, abrogation of Tal1 inhibition of E protein function was likely a major contributor to both the up-regulation of *p16(Ink4a)* and *p21(Cip1)* expression and impairment in cell cycle progression and proliferation. A previous study showed that retroviral transduction of mutant *N-ras* gene (*N-rasG13R*) into $CD34^+$ human cord blood cells resulted in up-regulation of *p16(Ink4a)* and *p21(Cip1)* expression in primitive $CD34^+$ cells in a dose-dependent manner and these cells produced a higher proportion of myelo-monocytic progenitors (Shen et al., 2007). Therefore, this suggests that higher expression of *p16(Ink4a)* and *p21(Cip1)* may also contribute to the faster rate of differentiation seen in $SCL^{\Delta/LacZ}$ cells.

While *p16(Ink4a)* transcription was inhibited by Tal1 in differentiating cells of this lineage, the *Gata2* gene appeared to be transactivated by Tal1. A region in intron 4 of *Gata2* was previously shown to have strong enhancer activity in endothelial and mouse fetal liver cells (Khandekar et al., 2007; Wozniak et al., 2007), and findings from our studies are compatible with it also being active in mononuclear phagocytes. Further, the slight acceleration of differentiation seen in *Tal1* knockout cells could be explained by the reduced levels of *Gata2*. A similar phenotype was observed in JMML cells where

mutant Shp2-expressing hematopoietic progenitors had low Gata2 expression and exhibited enhanced monocytic differentiation (Yang et al., 2009). Moreover, GATA2 expression in brown adipocyte precursors was down-regulated in a differentiation-dependent manner and deletion of *GATA2* gene resulted in enhanced differentiation (Tsai et al., 2005; Okitsu et al., 2007). *GATA2* deleted adipocytes showed up-regulation of several adipocyte-specific marker genes like *PPARγ, UPC-1* and *PGC1α* and mitochondrial genes like *COX II, COX IV* and *ATP-synthase* and morphologically showed higher intracellular lipid accumulation suggesting enhanced differentiation (Tsai et al., 2005). Therefore, these studies in myelo-monocytic leukemia cells and adipocytes suggested that *Gata2* is a key regulator of this process and proper timing of *Gata2* down–regulation may be critical for differentiation. In support of this concept, maintenance of high-level Gata2 expression in murine bone marrow cells blocked both their differentiation and amplification and suggested that regulation of Gata2 expression is a critical event in normal hematopoiesis (Persons et al., 1999). Our data suggest that a similar role of Gata2 could also explain the enhanced differentiation seen in $SCL^{\Delta/lacZ}$ cells and could have been brought about by an earlier decline in Gata2 expression in these cells.

Reduced *Gata2* expression could also contribute to the cell cycle defect observed in $SCL^{\Delta/LacZ}$ MM precursor cells. In G1ME cells, shRNA-mediated knock down of *Gata2* expression resulted in proliferative defect brought about by delayed entry from G1 to S phase of the cell cycle (Huang et al., 2009). Consistent with this cell cycle defect, expression of several genes including *E2f2* and *Skp2* was reduced while *Cdkn1a (p21)* and *Cdkn1b (p27)* expression was increased. More interestingly, *Gata2* knockdown

resulted in increased myeloid gene expression including *Mpo, PU.1, Hhex* and *Cebpa* suggesting that the cell cycle arrest could be indicative of enhanced myeloid differentiation (Huang et al., 2009). Our data suggest that *Gata2* reduction in $SCL^{\Delta/LacZ}$ cells may have similarly contributed to the cell cycle delay and enhanced differentiation of MM precursor cells (Fig. 11A) and might have also resulted in increased *Csf1r* gene expression (Fig. 15). Interestingly, and in contrast with *p16(Ink4a), Gata2* expression was not affected by *Tal1* over-expression and was reduced only with *Tal1* nullizygosity, reflecting, potentially, a difference in the quantitative requirement for Tal1 in genes activated and repressed by this transcription factor.

Although expressed throughout the process of differentiation, DNA and protein interaction of Tal1 may vary depending on the target gene and that, in turn, may closely regulate proliferation and differentiation of MM precursors. Our gene knockout data suggests that TAL1 acts both as a transcriptional activator and repressor, perhaps depending on the interacting partners and might switch from one role to the other in a stage-specific manner. Studies done in our lab have clearly shown this dual activity of Tal1 in erythroid cells. In MEL cells, Tal1 can activate the expression of cell surface protein 4.2 (P4.2) by associating with a multi-protein complex (consisting of E47, GATA-1, LMO2, Ldb1 and SSBP2/3) that interacts with two E box-GATA motifs in the promoter region of *P4.2* gene (Xu et al., 2003; Xu et al., 2007). Dimethyl sulfoxide (DMSO)-induced terminal erythroid differentiation of MEL cells results in gradual increase in both Tal1 and P4.2 expression. Nuclear extracts from differentiating MEL cells were analyzed by EMSA using a radiolabeled probe containing one of the E box-GATA motifs in *P4.2* gene promoter, and showed an increase in binding with

differentiation, which finally declined with terminal differentiation (Xu et al., 2003). In uninduced MEL cells, the SWI/SNF protein, Brg1 can also associate with the same Tal1-containing complex at the same site to repress P4.2 expression by recruiting the mSin3A and HDAC2 corepressor complex (Xu et al., 2006). On induction of erythroid differentiation of MEL cells, Brg1 gets dissociated from the complex and switches the complex from a repressor to an activator of *P4.2* gene expression. The Tal1-mediated repression of *P4.2* gene expression in uninduced cells can also be augmented by co-expression of Eto2 and Mtgr1 and both these proteins decrease with differentiation of MEL cells (Cai et al., 2009).

In MM precursor cells, while *p16(Ink4a)* gene expression increased, *Gata2* gene expression was found to decrease with differentiation and *Tal1* knockout resulted in up-regulation of *p16(Ink4a)* and down-regulation of *Gata2* expression. This suggested that Tal1 has opposite effects in regulation of these two target genes and given that direct Tal1-E protein association with gene regulatory regions was detected in earlier MM precursor cells (day 4, Fig. 17C), Tal1 may repress *p16(Ink4a)* expression and activate *Gata2* expression at this stage of differentiation (Fig. 19 i). In more mature cells (day 7, Fig. 17D), Tal1-E protein association at the same regulatory sites were near absent for the *p16(Ink4a)* gene and undetectable for the *Gata2* gene, in spite of continued *Tal1* expression (Fig. 3A and 3E). At this stage of differentiation, *p16(Ink4a)* expression was higher than the earlier stages suggesting that other E proteins may bind these regulatory sites at later times or independent pathways of *p16(Ink4a)* regulation exist (Fig. 19 ii). This also suggested that Tal1 may not be required for continued repression of this gene at later stages of differentiation. On the other hand, *Gata2* gene expression may be more

directly regulated by Tal1 with no binding detected in more mature cells that do not express Gata2 (Fig. 19 ii). Tal1-mediated active repression of *p16(Ink4a)* gene may have been relieved in $SCL^{\Delta/LacZ}$ cells where E2A homodimers or other E proteins might have bound the upstream E boxes and resulted in its higher expression (Fig. 19 iii). In $SCL^{\Delta/LacZ}$ cells, we detected a considerable decrease in *Gata2* expression that could be explained by the absence of Tal1-E protein complex at the intron 4 enhancer region. This region may be specifically and selectively bound by the Tal1-E protein heterodimer and not by E protein homodimers and hence could not drive *Gata2* expression in absence of Tal1. This suggested another interesting aspect in gene regulation that dictates binding specificity and sequence recognition by different complexes. In addition to up-regulation of CDK inhibitors, decline in Gata2 expression may also have added to the phenotype observed in $SCL^{\Delta/LacZ}$ cells. Further studies are required to identify the Tal1-interacting proteins and analyze the mechanism of its dual role in this lineage. Another interesting feature is the stability of the complexes formed, because *Tal1*-over expression did not change *Gata2* expression but did result in slight decrease in *p16(Ink4a)* expression. However, the modest decline in *p16(Ink4a)* expression in *Tal1*-over expressing cells could not sufficiently explain the high proliferative potential of these cells suggesting a different mechanism of action. Although *Tal1* over-expression resulted in increased *Csf1r* mRNA expression in M1 cells and primary MM precursor cells, preliminary ChIP analysis for Tal1 binding at the *Csf1r* regulatory regions could not identify its association with those regions (data not shown). Therefore, the mechanism for positive regulation of *Csf1r* gene expression by Tal1 will require further studies.

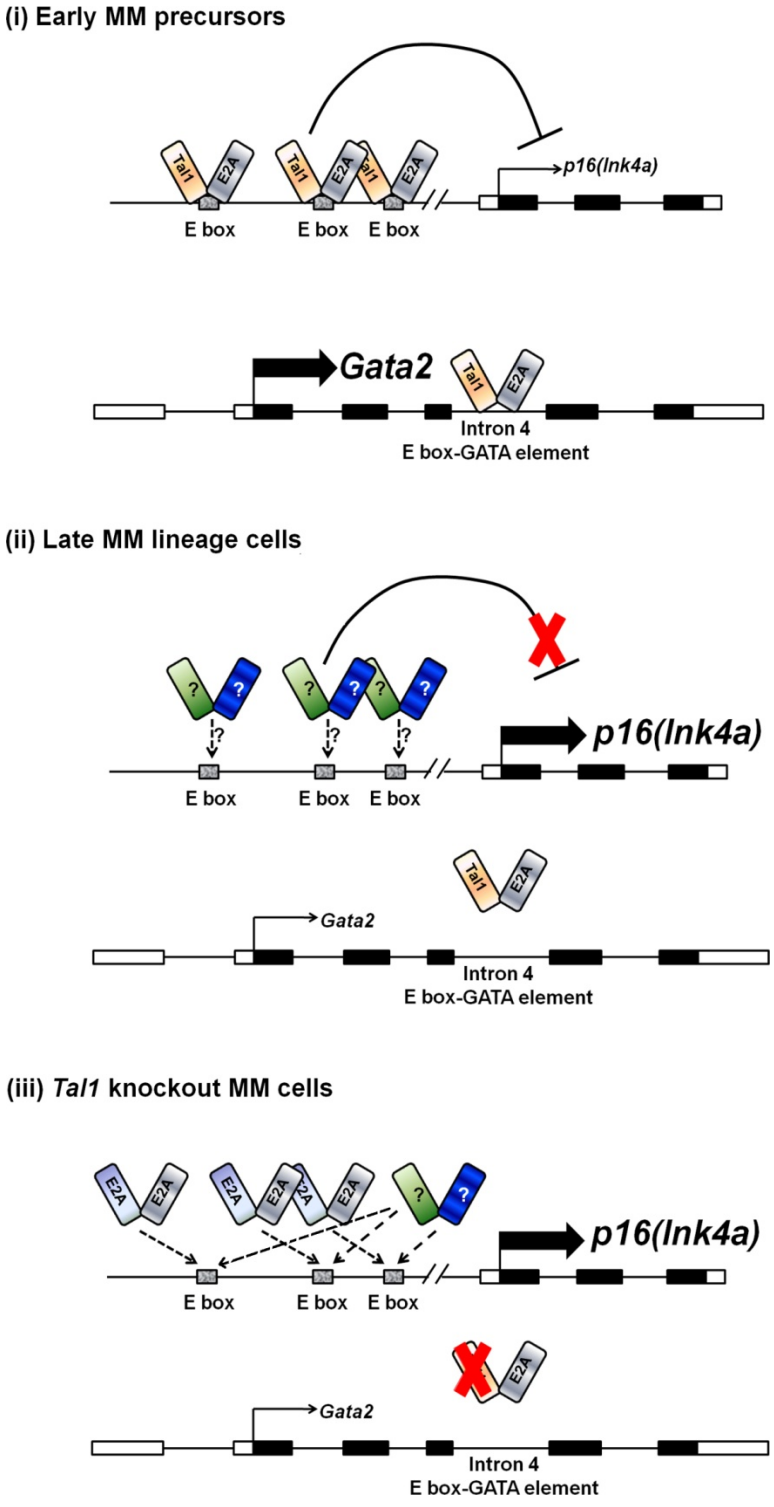

Figure 19. Model of gene regulation by Tal1 in MM precursor cells**.** Schematic representation of Tal1 binding and target gene regulation in early precursors **(i)**, late

monocyte-macrophage cells **(ii)**, and *Tal1* knockout cells **(iii)**. Higher expression is denoted by gene names in bigger fonts and thicker arrows.

The comparative cell proliferation studies in *Tal1*$^{+/+}$ (wild-type) cells and *Tal1*$^{+/-}$ cells showed that a 50% reduction in Tal1 expression resulted in a detectable difference in cell numbers at later stages of the culture period (Fig. 8C). This suggested that more mature cells may be more sensitive to changes in Tal1 expression. Interestingly, *p16(Ink4a)* expression in *SCL*$^{\Delta/LacZ}$ cells was maximally up-regulated at later times in culture (days 6 and 8, Fig.14C) although the ChIP studies did not detect Tal1 association at *p16(Ink4a)* upstream sequences at this stage. In M1 monocytic leukemia cells, IL-6-induced differentiation results in elevated expression of mRNA encoding dominant-negative HLH proteins, Id1 and Id2 along with loss of Tal1-E2A DNA binding (Voronova and Lee, 1994). Id1 expression is also closely associated with the mouse BM myeloid lineage cells and is highly expressed in Mac-1^{+} myeloid cells and in mononuclear cells of blood and peritoneum (Cochrane et al., 2009). In normal human myeloid cells, *Id2* mRNA has been detected in cultured macrophages from bone marrow and in mature granulocytes and monocytes from peripheral blood (Ishiguro et al., 1996). Preliminary studies done in transiently transfected cell lines showed that these dominant-negative HLH proteins are critical regulators of *p16(Ink4a)* and *p21(Cip1)* gene expression (Prabhu et al., 1997; Alani et al., 2001). The Id proteins can inhibit cellular senescence through repression of *p16(Ink4a)* expression, and *Id1*-null murine embryonic fibroblasts (MEFs) undergo premature senescence despite normal growth profiles at early passages (Alani et al., 2001). Id1 over-expression can also accelerate cell growth by inhibiting p21 expression, probably by counteracting the action of E2A that can activate

p21 expression and cell cycle arrest in G1 to S phase transition (Prabhu et al., 1997). Future investigation into the role of Id proteins, particularly at later stages, may enable better understanding of this process and shed more light on function of Tal1 in this lineage.

Tal1-null adult HSCs have impaired short-term repopulating ability, predominantly of the myeloid lineage, but no defects in long-term repopulation or self-renewal (Curtis et al., 2004). Tal1 is essential for the formation but not the maintenance of adult HSCs (Mikkola et al., 2003). A possible explanation of redundant function of Tal1 in adult HSC is expression of the related bHLH transcription factor, Lyl1. However, Lyl1 cannot rescue early lethality of *Tal1*-null mice despite similar patterns of embryonic expression (Giroux et al., 2007), and enforced expression of *Lyl1* in *Tal1*-null ES cells did not rescue their defective development (Porcher et al., 1999), suggesting that Lyl1 can not compensate for Tal1 in developmental hematopoiesis. In adult hematopoiesis, Lyl1 is expressed in myeloid and B cell lineages as well as HSCs but is dispensable for hematopoietic development. Transplantation assays with *Lyl1*-null HSCs showed defective lymphoid function but did not affect myeloid repopulation, suggesting that Lyl1 is also dispensable for adult HSCs (Capron et al., 2006). Repopulation analysis with HSCs from a *Lyl1/Tal1*-conditional double knockout mice showed a gene dose-dependent loss of hematopoietic progenitors due to apoptosis (Souroullas et al., 2009). A similar functional redundancy may also be possible in MM precursor cells. While $Tal1^{-/-}Lyl1^{+/+}$ CMPs showed a 3- to 4-fold upregulation in *p16(Ink4a)* mRNA expression, compared to control, almost 9- to 10-fold upregulation was noted in $Tal1^{-/-}Lyl1^{+/-}$ and $Tal1^{-/-}Lyl1^{-/-}$ CMPs (unpublished data, Dr. David Curtis, The Royal Melbourne Hospital, Australia),

suggesting that expression of at least this gene is regulated by both the transcription factors. Therefore, further studies of differentiation from *Tal1*$^{-/-}$*Lyl1*$^{-/-}$ MM precursor cells may reveal more severe defects and identify a different level of gene regulation in this lineage.

The DNA binding-defective mutant of Tal1 (Tal1^{T192P}) used in the rescue analysis is a well-characterized mutant (Huang et al., 2000) where the single threonine to proline substitution renders it completely unable to interact with DNA without effecting any other function of Tal1. Deletion of the basic region of Tal1 (ΔbTal1) has been previously used by other groups to study DNA-binding dependent functions of Tal1, but this mutant has the possibility of functioning as Id proteins which are HLH proteins lacking the basic region. Anyway, Tal1^{T192P} was unable to rescue the proliferative defect in *SCL*$^{\Delta/LacZ}$ cells, which suggested that DNA-binding ability of Tal1 is critical for its function in this lineage. Embryos homozygous for a DNA-binding mutant of Tal1 exhibited anemia at the yolk sac and fetal liver stages and showed presence of immature proerythroblasts suggesting a complete block or delay in erythroid maturation (Kassouf et al., 2008). Although these embryos survived the yolk sac stage and showed earliest hematopoietic development and commitment, almost 80% of embryos died at E14.5 primarily due to defective maturation of primitive erythroid cells. Therefore, DNA-binding independent function of Tal1 was sufficient for hematopoietic stem/progenitor cell specification but was not enough for terminal erythroid maturation. Our studies showed that similar to erythroid maturation, DNA-binding ability of Tal1 is also required in expansion and differentiation of MM precursor cells.

Expression of Gata2 was down-regulated in *SCL*$^{\Delta/LacZ}$ cells and re-introduction of the *Gata2* cDNA resulted in complete loss of cell viability within 24 hr. This suggests that timing of Gata2 expression may be critical in this lineage and non-redundant functions of Gata2 and Tal1 exist although expression of one may be regulated by the other. In osteoclasts, which originate from the same parental cells as macrophages, a sequential requirement of Tal1, Gata2, M-CSF and RANK-ligand has been demonstrated (Yamane et al., 2000). Gata2 is highly expressed in enriched populations of pluripotent HSCs (Orlic et al., 1995) and also in early erythroid cells (Leonard et al., 1993), mast cells (Dorfman et al., 1992), megakaryocytes (Visvader et al., 1995) and at early stages of CMPs differentiating towards MM lineage (Tagoh et al., 2002). Enhanced expression of Gata2 in BM hematopoietic progenitor cells using the same vector that we used for our study resulted in a 50%-80% reduction in colony formation compared to empty vector transduced cells (Persons et al., 1999). These cells remained viable but showed loss of spleen-colony forming ability and did not contribute to multilineage reconstitution of transplanted mice. It is possible that the loss of viability in *SCL*$^{\Delta/LacZ}$ cells transduced with *Gata2* cDNA could be due to a deleterious effect of Gata2 over-expression in a *Tal1*-null background. Nevertheless, complete recovery of *SCL*$^{\Delta/LacZ}$ cells transduced with a Tal1 cDNA suggested that the phenotypic consequences in *SCL*$^{\Delta/LacZ}$ cells were specifically due to Tal1 loss and not due to any indirect effects.

From this study I propose a novel role of Tal1 in a critical branch of hematopoiesis. To date this is the most comprehensive study of role of Tal1 in monocytopoiesis that identified critical functions that are regulated by Tal1 and also explained the molecular mechanism behind those functions. I identified two potential

targets of Tal1, p16(Ink4a) and Gata2, which are extremely critical not only for proliferation, cell cycle control and differentiation but also in macrophage function. Previous studies have suggested that Tal1-E protein binding at upstream E box sequences regulate expression of CDK inhibitors but none of these studies demonstrate endogenous binding of these factors at regulatory E box motifs. We could detect direct association of Tal1 and E protein at *p16(Ink4a)* upstream regulatory sites and also proposed a possible mechanism of regulation at different stages of monocytopoiesis in primary BM cells. Expression of *Gata2* was also shown to be regulated by the intron 4 enhancer in MM precursors similar to previous findings in embryonic endothelial and hematopoietic cells. Therefore, our study probably adds another level of control in this cell lineage and explains how the regulators are being regulated. Although we concentrated on the physiological role of Tal1 in growth and differentiation of monocytes into macrophages, further study to investigate its role in macrophage lineage may also be interesting. *In vivo* macrophage depletion by injection with liposomal-encapsulated clodronate (LIP-CLOD) induced megakaryocytic and thrombopoietic activities (Alves-Rosa et al., 2003), reduced BFU-E numbers and circulating reticulocytes (Giuliani et al., 2001) and produced erythroid abnormalities (Giuliani et al., 2005; Lisa Giuliani et al., 2007). Whether the function of Tal1 in macrophages is related to formation of erythroid blood islands in BM warrants further investigation and might explain the *in vivo* role of Tal1 in this lineage.

Taken together, our studies elucidate a novel physiological role of Tal1 that may also be involved in hematopoietic disorders. It also suggests molecular mechanisms of the function of this transcription factor and identifies novel target genes and suggests new

cellular and biological functions that may be regulated by this important bHLH transcription factor.

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